# THE SPERMATHECA OF *EURYCEA BISLINEATA*.[1]

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Since 1785 it has been known, first through Spallanzani, that fertilization in salamanders is internal. Later there were found at intervals in several species "receptacula seminis" into which spermatozoa are received and stored. So far as is known in most species of salamanders the spermatozoa are transferred some time before egg-laying to the cloaca of the female by means of spermatophores deposited by the male and received by the female either actively or passively, the method of deposition and reception varying somewhat in the few species which have been noted at breeding.

The "receptacula seminis" or spermathecæ[2] of several forms has been noted by Rathke (1820), Leydig ('53), Siebold ('58), Jordan, Fisher and Stieda ('91), and Kingsbury ('95). The work of Dr. Kingsbury is especially important as a comparative study of the spermathecæ of *Necturus maculatus*, *Diemyctylus viridescens*, *Desmognathus fuscus*, *Amblystoma punctatum*, *Plethodon glutinosus* and *Spelerpes bislineatus* (*Eurycea bislineata*). In all of these forms the spermatheca is dorsal to the cloaca and consists of a number of tubules. These tubules open severally into the dorsal wall in *Necturus*, *Diemyctylus*, and *Ambystoma* and cover comparatively large areas in this region. In *Desmognathus*, *Plethodon* and *Eurycea* the organ is more compact and opens by a single central tubule into the cloaca.

Kingsbury describes but one specimen of *Eurycea bislineata* taken in October and containing no spermatozoa. It is the purpose of this paper to continue the work on *Eurycea*, describing the mature spermatheca at various seasons and in the course of its development.

[1] Contributions from the department of Zoölogy, Smith College, No. 131.

[2] Dr. H. H. Wilder calls attention to the fact that the correct word is Spermatotheca, using the root of the word (spermato-) instead of the nominative stem.

## I.

## The Mature Spermatheca.

The spermatheca of *Eurycea bislineata* is a compact, definitely bounded organ. Lying in a median dorsal position with reference to the cloaca it extends from the opening of the organ, a point cephalad to the vent of the cloaca, to the posterior wall which lies above the vent of the cloaca. A mature spermatheca is usually from 1/2 mm. to 2/3 mm. in antero-posterior extent, approximately as wide as it is long and slightly higher, dorso-ventrally, than it is wide—that is, somewhat conical in shape.

The spermatheca is a sac of tubules. The storage tubules, where the spermatozoa lie in season, are flask-shaped, from seven or eight to sixteen in number, and they converge into one central tubule which bends abruptly and opens into the cloaca. The sac is of heavily pigmented connective tissue. In the posterior wall and the floor of the sac this is thickest, most coarse and black, and the flask ends of the tubules are imbedded in the mass as in sockets. The coarse, pigmented tissue thins out in the anterior region and there is none of it in proximity to the necks of the flask-shaped tubules or the central tubule.

The extreme caudal end of the sac is but loosely connected with the cloacal wall and surrounding tissues, being held in place by scattered coarse strands of connective tissue. But approaching the mouth of the organ, where the pigmented floor thins out, it becomes more a part of the cloacal structure, the smooth musculature of the cloacal walls merging with the similar fibers of the interior of the spermatheca.

The arrangement of the tubules is noteworthy. The flask-shaped ends lie in the floor of the sac imbedded in pigmented tissue. Some are quite anterior, being anterior to the plane of the opening of the central tubule into the cloaca. But these most anterior tubules, one on each side of the median line, have their sockets of pigmented tissue though pigment is otherwise sparse in this region. The necks of the flasks converge in the posterior end of the sac and form the central tubule which runs dorsally anterior. Thus some of the flasks have very long and narrow necks. The convergence of the necks occasions a large reservoir which narrows into the central tubule proper.

The lining of the whole system of tubules is of most delicate columnar epithelium which extends into the cloaca in the region around the mouth of the central tubule. It is a markedly different tissue from the mucous lining of the cloaca. There is no mucous secretion from the spermatheca; nor at times is there any secretion of any kind as far as can be ascertained by staining reactions. Save for the spermatozoa during the breeding season the lumina may be clean and clear. Some series, however, show a faint indefinite substance, rather stringy, in the flask ends. The necks and central tubule may show slight traces of the matter also, though it is rare in the central tubule which is generally unusually clear.

There is a slight differentiation in the lining tissue in different parts of the system. In the blind ends the cells are long. The bases are densely granular with large nuclei. The apices stain faintly; they seem filled with minute, transparent globules. The apices are irregular—they do not form an even border for the lumen. They give a picket-fence appearance. In some series these inner halves of the cells are shrunken so that their ends appear as amœba-like processes, or as if they were in an exhausted state. Yet these conditions occur during the fall and winter months during which it is to be supposed that there is no reason for activity of the cells as no spermatozoa are present.

The cells of the necks of the flask tubules are shorter than those of the blind ends and produce a much more even border—that is, there is no appearance of shrinkage of the cells. The apices are rounding. It is in the central tubule, however, from the region of the convergence of the necks to the area surrounding the mouth of the cloaca that there is the most marked difference from the lining of the flask ends. The appearance of the central duct is constant at all seasons. These cells do not differ as to bases but the apices are very slightly longer and stain even more delicately than do those of the flask ends—there is no globular appearance. The apices fit perfectly together and form a smooth, even surface for the lumen. No variation of this condition has been noted in any mature spermatheca.

The size of the lumen of the central tubule varies—it is a reservoir at the convergence of the necks. In what are presumed

to be very old animals there are two of these reservoirs formed by the necks of each side; these join, narrow and become the central tubule. The lumen of this tubule near the opening into the cloaca is often very small—about 1/100 mm.—but it may be as wide as 4/100 mm. The lumina of the necks vary around 1/100 mm. and those of the flask ends are usually about 6/100 mm. in diameter.

The dense coat of pigmented tissue, covering and binding in place the tubules, has been spoken of. This coat is thin, sometimes of but a few strands on the roof of the spermatheca. It is along this dorsal region that the central duct lies. This duct and the necks are surrounded by massed smooth muscle fibers which do not differ from the smooth muscle of the walls of the cloaca.

Unless injected, it is difficult in amphibian material to study the blood supply of an organ. Red blood corpuscles stain vividly with eosin and when present in numbers indicate blood vessels and capillaries even when dense pigment is present. In one series of a spermatheca which is on the verge of maturity many blood corpuscles have remained in this region. It is evident that the blood supply is rich. Corpuscles running single file everywhere throughout the region of the spermatheca indicate a close network of capillaries and some larger blood vessels are present. Only injection would show certainly, however, just where these vessels branch from the dorsal aorta which lies immediately above the spermatheca.

The mating of *Eurycea* takes place in the spring. There have been no spermatozoa found in the spermatheca during the fall and winter months. April may be taken as the average mating season though there must be as variable a season for mating as there is for egg-laying and many early and late dates have been recorded for *Eurycea* eggs.

Spermatozoa are found in the flask tubules usually in dense, orderly whorls similar to the groups found in the vas deferens of the male. They may be, however, scattered and tangled. No spermatozoa have been found in the central tubule in any series I have sectioned and in but one are there any in the necks of the tubules. This is a sagittal series and some sections,

in one side of the spermatheca, pass longitudinally through a flask and its neck. The spermatozoa are streaming in a mass from the flask into the neck. But other sections show that they have not proceeded as far as the central duct.

Flask tubules containing spermatozoa show a modification of the epithelium. The tall columnar cells are unrecognizable. There is instead a narrow row of flat cells utterly different in appearance from columnar cells. The delicate part of the cells has completely disappeared and no ragged nor shrivelled edges even indicate its former presence. The width of these cells is 1/100 mm. in comparison with measurements of 3/100 and 4/100 mm. during the non-breeding periods.

Some of this difference might be accounted for by presuming that the tubules are stretched by the mass of spermatozoa contained within them. However, there does not seem to be any such stretching. Measurements of the diameter of the tubules during the breeding season are normal corresponding to the size of the spermatheca. Also when the flasks are cut squarely through, the whorl of spermatozoa is shown lying in the lumen with no adherence to the walls. There is no evidence of crowding or packing.

## II.

## Development.

The above description is of a typical mature spermatheca following at least one season of egg-laying. The organ in some specimens which are larger and presumably older differs in some details and will be discussed at the end of this part on the development.

The development of the spermatheca is to be considered with reference to the growth of the cloacal region also. There is a great deal of change in the whole region from the time of metamorphosis to sexual maturity.

The youngest specimen prepared was a 45 mm. female identified by Mrs. Wilder as a stage in early metamorphosis. There is no indication of any spermathecal tubules though the beginning of some cloacal glands may be apparent. In this paper the term "cloacal glands" is applied to the mucous glands of the

cloaca. Kingsbury speaks of "ventral glands" but since many of the tubules are in the lateral walls of the cloaca and far dorsal, some beginning near the walls of the spermatheca, the more general term "cloacal glands" may be accurate enough. The cloaca at this stage is very simple; the walls are smooth and the only projection or fold is a small median papilla.

Other young animals prepared are 58 mm., 59 mm., 64 mm., 66 mm. and 68 mm. total length. But after metamorphosis there is no definite guide to the age of the animals as length is not a criterion and the size of the gonads is variable in all ages at various seasons, and within this range of lengths mature organs are found. A 67 mm. female killed in April was functional with spermatozoa in the flask tubules. A 68 mm. animal killed in June had a mature spermatheca which undoubtedly had been functional at least during the recent breeding season. Another April specimen, 69 mm. long, contained spermatozoa. Roughly speaking, size up to 68 or 70 mm. indicates sexual immaturity. Animals longer than 70 mm. will generally have the mature organ. Exceptions must be numerous considering the variable length of individuals at metamorphosis (I. Wilder '24).

In a 58 mm. animal identified by Mrs. Wilder as in advanced metamorphosis there are four distinct tubules in the dorsal region of the cloaca. They are in pairs—two on each side of the median line and one pair posterior to the other. These tubules are made up of a very small group of cells closely grouped and darkly stained with barely perceptible lumina and short ducts. The ducts very nearly reach the thick walls of an invagination of the cloacal wall which is the anlage of the central tubule. Several smaller but otherwise similar groups of cells appear more ventral but are most probably not part of the spermatheca group.

A 59 mm. animal in the incipient metamorphic stage also shows two pairs of tubules, one posterior to the other. Although this animal is not as far advanced in metamorphosis as the 58 mm. individual, the spermatheca anlage is in a more advanced condition. The lumina of the tubules are definitely open, the necks curve somewhat dorsally toward the median line and reach the walls of the central tubule. The opening of the central

tubule into the cloaca is minute and the very short duct above is broader in diameter. Columnar epithelium is already present lining this little chamber. There is no sign of the columnar epithelium in the necks or dense cells of the two primary tubules.

A 64 mm. animal, an adult probably recently transformed, shows another pair of tubules and otherwise little change in the spermatheca development, though the cloacal glands are greater in number and length and the cloaca is beginning to form folds of the walls.

In a 66 mm. animal there is another pair of tubules thus making four tubules on a side. The tubules seem to be all of the same size; the blind ends show an open lumen; the necks are solid cords. The central tubule is no larger in size and extent that it was in the 58 and 59 mm. animals but it is further removed from the cloacal wall—more dorsal. There seems to be no opening at all into the cloaca. It is not probable that any sections are lost in this region which might account for not finding an opening. The organ at this time is functionless and is developing slowly; on the other hand the cloaca is developing steadily, that is, the walls are becoming folded and glands are enlarging. The spermatheca group of tubules is left behind until the approach of sexual maturity.

A 68 mm. animal was sectioned horizontally and badly torn by inability to fix the contents of the rectum, but there is discernible the same primitive condition as of the 66 mm. female. The number of tubules has increased; five pairs are certain but six may be present. All are in the same stage. In all these series there is no way of determining the primary pair of tubules nor the latest formed.

From the first few rudimentary tubules with their cord-like necks and small central tubule and the whole without any sign of pigment, to the pigment-walled mature spermatheca which occurs in so many 68–72 mm. females and in practically all of them of greater length, seems a great jump and implies rapid growth in a short period most probably just preceding the first egg-laying season. The author regards it as a piece of good-luck to have found, simply by chance sectioning, as there seems to be no other method of determining age at this period, a series

which is intermediate between the young and the mature spermatheca. This was a 71 mm. female caught and killed in September.

In this spermatheca the average number of tubules found in a mature organ is not complete (though at least one functional organ in this study was observed with no more) and only one pair, one tubule on each side of the median line, is mature in size and cell structure. The necks of these two largest tubules are well developed: the lumina appear at first sight to be open but upon higher magnification they are seen to be so filled with the faintly stained apices of the columnar cells as to be virtually closed tubes. The remainder of the tubules and their necks are in various stages of development.

A most marked development of the central tubule is evident. This seems much out of proportion to the same duct in the fully mature organ. The extent is less, that is, the tube is short and does not extend far posterior, but the diameter is wide. Above the short, stout entrance the duct immediately widens into a very broad chamber into which the necks empty. Most of the necks being rather immature their confluence is not so graceful as is typical in older spermathecæ. Also the cells of the columnar lining of the central tubule have not the length and delicacy of apices peculiar to this duct in all later stages, and consequently they are not so conspicuous.

Lastly pigment is present in quantities but not in the arrangement of the mature organ. Sockets around the ends of the two largest tubules are being formed. Around some of the smaller tubules also it has gathered in tangled networks, but around the largest pair it is more concentrated—coarse, thick strands. Aside from these foci the pigment is scattered and the pigment producing cells still visible. The definitely bounding walls of the organ are not formed.

The spermatheca just described measures slightly less than 1/3 mm. in length and must suffice to complete the developmental series. But a word may be said about the largest spermatheca observed during this investigation. The animal was a 95 mm. female killed in September. Unfortunately for the series as a whole it was sectioned horizontally but the spermatheca is perfect. Eighteen tubules were counted and, although

there is no apparent symmetry in the bilateral arrangement of the tubules, the necks from each side converge separately which causes two reservoirs and these, joining, form the large chamber of the central tubule. This narrows abruptly into the long, narrow dorsal passage which dips into the cloaca.

In this largest spermatheca as in some others which are presumed to be several breeding seasons of age, the central tubule extends quite to the posterior wall of the organ and, coincident, are the exaggerated curves of the necks of the flask tubules. All of the tubules lie in the floor of the organ but the most anterior have their blind end walls faced anteriorly. The necks then curve posteriorly and dorsally. The epithelium in this oldest series is particularly conspicuous and the whole thing is an organ of striking beauty, in size approximately 2/3 mm. long.

## III.

### Relation to Cloaca.

The development of the cloaca from the simple smooth-walled stage of larvæ to the complications of sexual maturity cannot well come within the scope of this paper, but a brief summary and a short description of the mature conditions should not be out of place in explaining some of the relations of the spermatheca.

The most distinctive feature of the cloaca after metamorphosis, aside from the development of the mucous glands, is the formation of a short, dorsal papilla midway between the vent and the opening of the oviducts. This lengthens antero-posteriorly becoming a fold and also deepens until it appears in sections like an icicle pendent from the roof of the cloaca. Until a period shortly preceding sexual maturity the walls of this fold are comparatively smooth. Then they become folded and especially modified at the tip. The tip broadens into a flat surface with two wings which extend close to the lateral walls of the cloaca so as to form two virtually closed dorsal passages through the cloaca up to the opening of the spermatheca where the entire fold ends. The fold undoubtedly functions as an egg guide causing a kind of continuation of the oviducts into the cloaca, but the function of the two small blind pouches in the posterior region of the fold is unknown.

Although deep folds and spurs in the walls of the cloaca admit of great expansion of the region it does not seem possible that more than one egg could pass at a time, and during the progress of this egg down one side of the cloaca corresponding to the oviduct from which it has proceeded the other passage must be entirely occluded by the pressure of the central fold against the opposite wall. Thus each egg after being squeezed through this crowded region of the cloaca arrives in the larger, freer space at the opening of the spermatheca. The distance from the opening of the oviduct to the mouth of the spermatheca is not over one half a millimeter.

The posterior wall of the fold and an extended area of the dorsal wall of the cloaca surrounding the opening of the spermatheca is lined with the delicate columnar epithelium otherwise singular to the central tubules of the spermatheca. The peculiar function of these cells can be guessed at; they secrete a substance which attracts spermatozoa. During egg-laying the smooth musculature of the spermatheca is probably affected by the convulsions of the entire region and spermatozoa are forced out from the necks of the flasks into the central tubule. Thus disturbed they may swim about and into the cloaca within the secretions of the columnar cells, however, thus preventing any loss. This secretion, as has been stated before, seems not to be mucin or any staining substance but is probably acid. Pfeffer has shown that malic acid is a common attraction for spermatozoa in ferns and Jordan ('91) believes it must be responsible for the attraction of spermatozoa to the spermatheca.

But the substances surrounding the egg as it is released into this region of the cloaca must in some manner offer greater attraction and the egg is probably surrounded with a numerous gathering of spermatozoa. It is not known whether one of these will penetrate the egg membrane immediately before it leaves the cloaca, or whether the whole group will promptly be expelled from the vent, one sperm gaining entrance and the rest perishing during attachment. There is no record of newly laid eggs being examined for impotent spermatozoa adhering to them.

The theory of the attraction of the columnar cells of the area around the mouth of the spermatheca also accounts for the

spermatozoa being drawn up into the spermatheca at the time of reception instead of attempting the larger passages of the cloaca, oviducts or even rectum.

## IV.

## Summary.

The spermatheca of *Eurycea bislineata* is a compactly walled-in set of tubules in the arrangement of a miniature "cat-o'-nine-tails"; the tails are the bulbed or flask-like storage tubules and the long curved handle is the central tubule which is both entrance and exit for spermatozoa. The organ develops slowly in the median dorsal wall of the cloaca during and after metamorphosis until the autumn before the first egg-laying season, when, more rapidly, the majority of the tubules are formed, pigment appears forming the walls and sockets for the tubules and the columnar lining of the system is completed, proliferating back, probably, from the central tubule.

The author expresses profound appreciation for the help and advice of Dr. Harris Hawthorne Wilder and Mrs. Inez Whipple Wilder in the preparation of this paper.

## Material and Technique.

Aside from dissections of fresh and preserved material, fifteen female *Eurycea* were sectioned. In size these vary from 45 mm. to 95 mm. total length. Specimens were killed in September, October, November, February, April and June. All were decalcified and sectioned entire—from the ovary to a point caudal to the vent of the cloaca—to preclude any distortion of the cloacal region. Bouin's fixative was generally used though some of the young animals were preserved in formalin. Fourteen were stained with hematoxylin-eosin and one with muci-carmine. Most of the series are transverse; two are frontal and two sagittal. The drawings are made with a projection apparatus.

### Abbreviations Used.

| | | | |
|---|---|---|---|
| *ant* | anterior | *nc* | nerve cord |
| *ap* | anterior pouch | *nt* | notochord |
| *bl* | bladder | *ov* | oviduct |
| *cg* | cloacal glands | *post* | posterior |
| *cl* | cloaca | *pu* | pubis |
| *ct* | central tubule | *pw* | posterior wall of spermatheca |
| *dor* | dorsal | *rc* | rectum |
| *f* | dorsal fold of cloaca | *sp* | spermatheca |
| *ft* | flask tubule | *spz* | spermatozoa |
| *ms* | mesonephros | *ur* | ureter |
| *n* | neck | *vent* | ventral |

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## EXPLANATION OF FIGURES.

### Plate I.

Fig. 1. (Mag. × c. 85.) Fig. 1 is a diagrammatic drawing of a young functional spermatheca with part of the pigment sac removed. The diagram is based upon the study of the spermatheca of the 69 mm. female of Fig. 3 and the immature spermatheca of a 71 mm. specimen described in the text. An average of eight tubules would be typical of this stage. The necks bend but slightly posterior and some are anterior in direction. At their convergence the central tubule is extremely large but short, antero-posteriorly, and narrows as it dips into the cloaca. The posterior wall and floor of the spermatheca are densely pigmented and sockets enclose the bulb of each flask tubule.

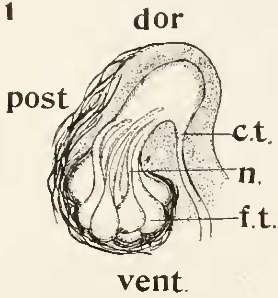
Fig. 2. (Mag. × c. 85.) Fig. 2 is a diagrammatic drawing of an older spermatheca. The sac wall is removed and half of the tubules are shown, bisected to show their contents—the coiled spermatozoa in the flask tubules. These tubules lying in the floor of the sac in pigmented sockets bend posteriorly and dorsally to converge into the central tubule. The reservoir formed by this convergence narrows and the duct passes anteriorly through the dorsal part of the sac where it bends to pass into the cloacal wall. The diagram shows this portion of the central tubule anterior to all the flask tubules but in many cases it passes between the two most anterior flask ends. See Fig. 7. (Mag. × c. 85.)

Fig. 3. (Mag. × 19.) Fig. 3 is a para-sagittal section through the region of the rectum and cloaca of a 69 mm. female to show the orientation of the spermatheca. The plane of sectioning is not quite true so that the anterior region of this drawing is very nearly median-sagittal showing the nerve cord, notochord, rectum and bladder. The posterior half of the drawing shows the end of the mesonephros, one oviduct, the spermatheca and the cloaca almost obscured by the many folds of its walls. It is to be noted in this figure that the mesonephros does not extend as far posterior as the spermatheca. This may be compared with transverse sections shown in other figures. In some individuals the mesonephros lies dorsal to the spermatheca and in others it ends more anteriorly. It is not certain whether these variations are due to changes or varying lengths in the mesonephros itself or if they might be due to the influence of the spermatheca.

An "*f*" placed below a fold separating the oviduct from the lower part of the cloaca is part of the fold represented in Fig. 10, transverse section.

The spermatheca in this specimen is young, of the type represented in Fig. 1. It is higher, dorso-ventrally, than it is long, antero-posteriorly. There are few tubules and the necks of these do not bend posteriorly. Through a median section the central tubule is large as in Fig. 1, but this section is through one side of it. The flask ends are filled with spermatozoa and in this section is the tubule described in the text from which the spermatozoa are streaming into the neck. The lining epithelium of these tubules is flat, unlike the delicate columnar cells pictured in the one flask tubule of Fig. 9, or in Fig. 5.

"*ap*" on this figure indicates an interesting fact about the mature cloaca that is very puzzling in transverse sections through the region—an anterior pouch, although this section is only through the wall of it. The anterior pouch is a ventral region of the cloaca separated from the anterior dorsal region by a band of muscle between the bladder and the cloaca. The region is left blank in the figure and indicated by "*m.*" Ventral dissections of the cloaca did not show this particular and in transverse sections it appeared so startling that its function was not guessed.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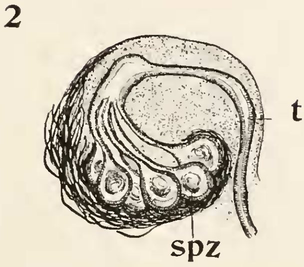
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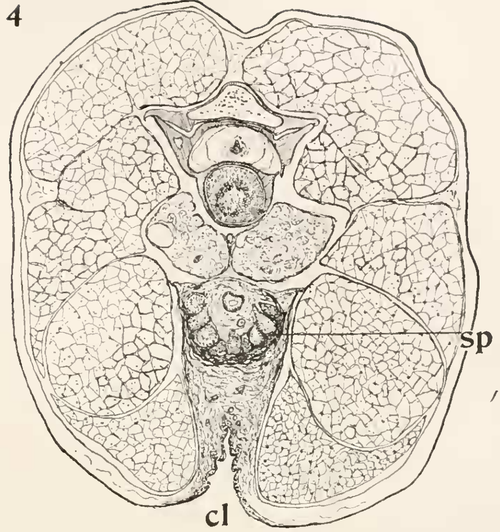
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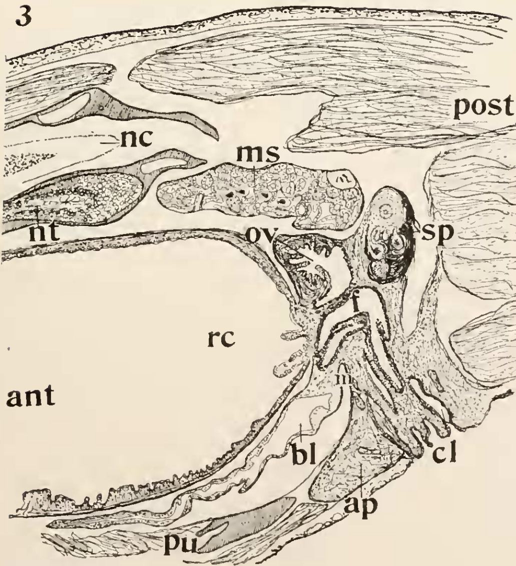
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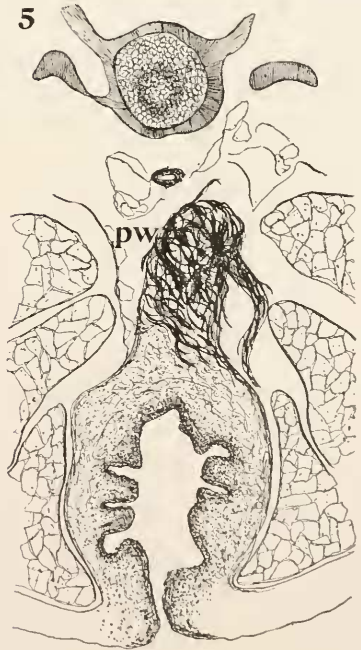
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From a sagittal view, however, it is very simply explained. During egg-laying the rectum and bladder are pushed anteriorly, the folds of the cloaca smooth out and the anterior pouch is drawn down as the vent is stretched open. It is but another rather larger fold of the cloaca.

FIG. 4. (Mag. $\times$ 17½.) Fig. 4 is a transverse section through a 68 mm. female midway through the spermatheca. The mesonephros lies above the spermatheca. In the floor of the spermatheca lie the flask tubules. A few necks are evident. The central tubule is near the dorsal wall. This is an animal killed in June; the tubules are empty and the columnar cells long. Although this animal is short in body length the spermatheca and cloaca show the maturity of several seasons of breeding.

PLATE II.

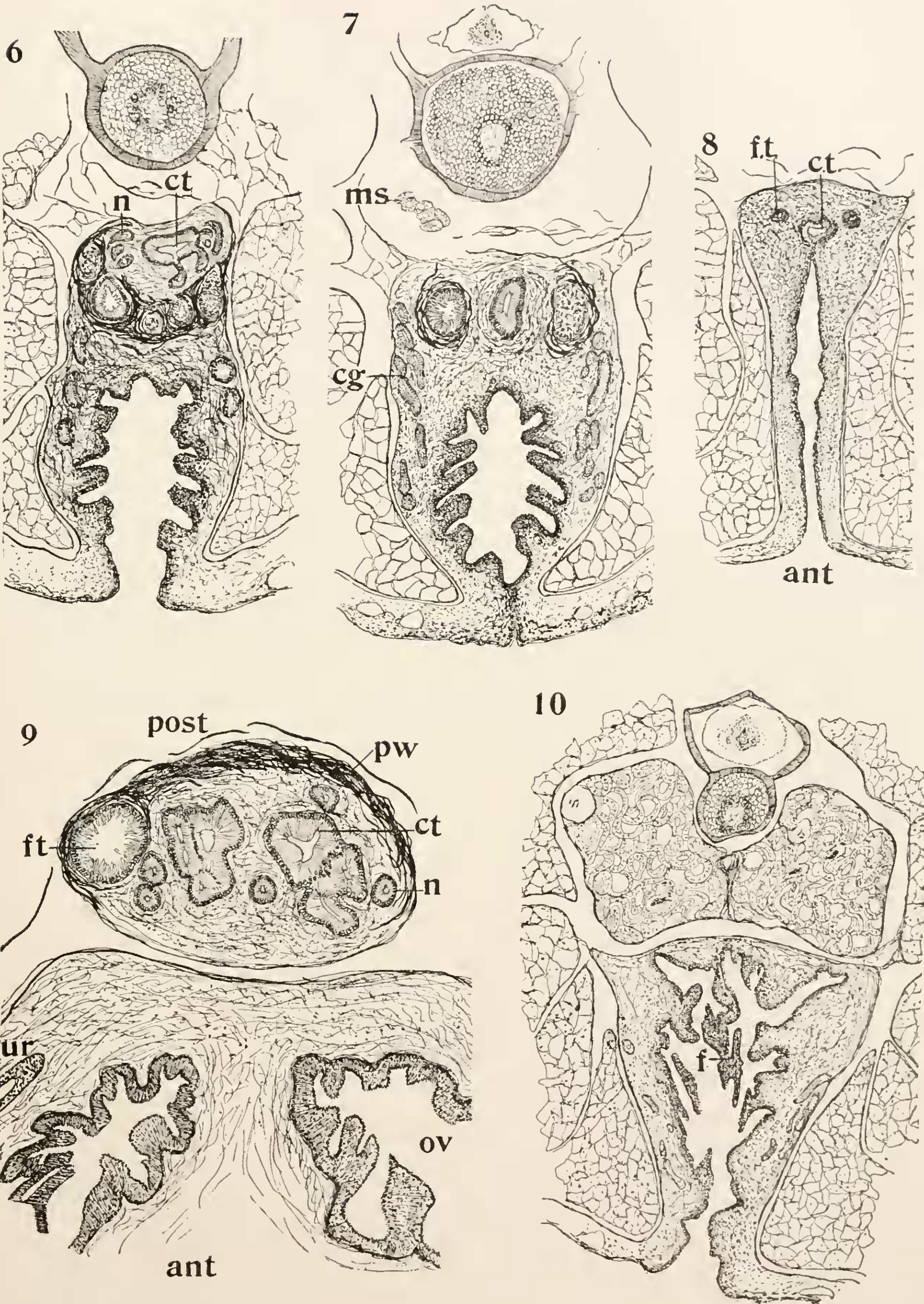
FIG. 5 (mag. $\times$ 40), FIG. 6 (mag. $\times$ 41½), FIG. 7 (mag. $\times$ 42). Figs. 5, 6 and 7 are regions of the spermatheca of a 70 mm. female. This organ has not as many tubules as that of the 68 mm. spermatheca of Fig. 4 nor is the cloaca as complex. On the other hand, there are more tubules and more pigment than in the 69 mm. spermatheca of Fig. 3. The significance of these variations after maturity is not known.

Fig. 5 is a transverse section through the posterior wall of the spermatheca. Fig. 6 is through the region where the necks are converging into the central tubule. Fig. 7 is through the anterior part of the organ; the central tubule bends to open into the cloacal wall and the most anterior flask tubules lie on either side.

FIG. 8. (Mag. $\times$ 50.) Fig. 8 is a transverse section through the anlage of the spermatheca in a 59 mm. female. One pair of flask tubules is shown and the central tubule in which the columnar epithelium has developed. The short, cord-like necks do not show in this section.

FIG. 9. (Mag. $\times$ 86.) Fig. 9 is a frontal section through a 95 mm. female. The necks converge into two groups which join to form the central tubule. One flask tubule is shown with the characteristic ragged-looking but nevertheless very delicate epithelium. The necks are similar to the epithelium of the central tubule. The oviducts and one ureter are in the anterior part of the drawing.

FIG. 10. (Mag. $\times$ 26½.) Fig. 10 is the region through the cloaca of the 68 mm. individual of Fig. 4 several sections anterior to the opening of the spermatheca. The central fold pendent from the wall of the cloaca divides the dorsal region, and serves as an egg guide.




VERA KOEHRING.

# THE INHERITANCE OF A MACULA MUTATION CONCERNED WITH ELYTRAL SPOTTING AND LATENT TRAITS IN THE MALE OF *BRUCHUS*.[1]

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A conspicuous, bilateral, three-character pattern is found on each elytron of the normal wild type female. It consists of two circular black spots placed one anterior and the other posterior, and a thin horizontal line of white pubescence along the inner edge. The elytron of the wild male is unmarked, the four black spots and the two white lines being absent. This condition enables the sexes to be easily distinguished and makes the wild *Bruchus quadrimaculatus*, Fabr., sex-limited.

In this culture, on October 3, 1922, at the University of Oklahoma, there appeared a male insect bearing indistinct black spots and white pubescence, a pattern similar to that of the female described above although less perceptible. This mutation was designated "macula." Approximately a year of inbreeding and selection was required before homozygous macula cultures could be assuredly separated from the wild type in which they originated, because the macula female and the wild female are identical in both homozygous and heterozygous cultures. The difference between the unmarked wild type male and the spotted, macula male was therefore the only visible criterion available for these selection tests.

The macula mutation is represented homozygously by the genes, MM, and its recessive allelomorph, spotted females and non-spotted males of the normal insect, homozygously by the factors, mm.

In the first test a (MM) female, homozygous for spotted males and females was crossed with a normal (mm) male, homozygous for non-spotted males and spotted females. The offspring from

[1] It is a pleasure to acknowledge my indebtedness to Dr. Frank R. Lillie, Director, for the privilege of utilizing the many excellent facilities of the Marine Biological Laboratory.

22 separate pairs, totaled 422 heterozygous, spotted (Mm) females and 412 heterozygous, spotted (Mm) males. This proves that the macula trait, spotted males and females, is dominant to the normal character, non-spotted males and spotted females. Complete data for this test is tabulated in Table I.

TABLE I.

$P_1$: 1 MM female, homozygous for spotted males and females × 1 mm male, homozygous for non-spotted males and spotted females.

$F_1$: Offspring:

| Pair No. | Females Spotted. | Males Spotted. | Pair No. | Females Spotted. | Males Spotted. |
|---|---|---|---|---|---|
| | (Mm) | (Mm) | | (Mm) | (Mm) |
| 3....... | 15 | 16 | 98........ | 15 | 15 |
| 2....... | 20 | 29 | 99........ | 32 | 42 |
| 17....... | 28 | 14 | 104........ | 19 | 9 |
| 19....... | 33 | 19 | 110........ | 1 | 1 |
| 36....... | 5 | 17 | 116........ | 6 | 5 |
| 44....... | 38 | 32 | 5........ | 13 | 10 |
| 51....... | 11 | 13 | 9........ | 0 | 3 |
| 54....... | 24 | 27 | 10........ | 10 | 0 |
| 59....... | 24 | 38 | 106........ | 19 | 18 |
| 60....... | 25 | 16 | 112........ | 27 | 35 |
| 94....... | 18 | 14 | 120........ | 34 | 39 |
| | | | Totals...... | 422 | 412 |

TABLE II.

RECIPROCAL CROSS.

$P_1$: 1 mm female, homozygous for non-spotted males and spotted females × 1 MM male, homozygous for spotted males and females.

$F_1$: Offspring:

| Pair No. | Females Spotted. | Males Spotted. | Pair No. | Females Spotted. | Males Spotted. |
|---|---|---|---|---|---|
| | (Mm) | (Mm) | | (Mm) | (Mm) |
| 1....... | 11 | 8 | 79........ | 31 | 34 |
| 10....... | 1 | 2 | 80........ | 37 | 29 |
| 34....... | 15 | 30 | 81........ | 33 | 16 |
| 39....... | 11 | 11 | 88........ | 18 | 16 |
| 47....... | 24 | 19 | 90........ | 24 | 23 |
| 48....... | 3 | 6 | 92........ | 51 | 56 |
| 49....... | 15 | 9 | 109........ | 19 | 27 |
| 63....... | 28 | 46 | 123........ | 22 | 25 |
| 64....... | 6 | 7 | 6........ | 2 | 0 |
| 68....... | 7 | 6 | 9........ | 9 | 5 |
| 69....... | 15 | 16 | 7........ | 49 | 27 |
| 78....... | 44 | 46 | 8........ | 12 | 9 |
| | | | Totals.... | 488 | 483 |

TABLE III.

$F_1$: Mm heterozygous spotted female × Mm heterozygous spotted male.
$F_2$: Progeny:

| Pair No. | Females Spotted. | Males. | |
|---|---|---|---|
| | | Spotted. | Non-spotted. |
| | (MM, 2 Mm, mm) | (MM, 2 Mm) | (mm) |
| 4 | 23 | 19 | 9 |
| 87 | 37 | 30 | 7 |
| 126 | 15 | 10 | 3 |
| 127 | 45 | 33 | 15 |
| 128 | 30 | 19 | 4 |
| 11 | 14 | 16 | 4 |
| 3.1 | 26 | 15 | 6 |
| 3.4 | 7 | 6 | 1 |
| 3.7 | 48 | 31 | 13 |
| 19.1 | 9 | 11 | 3 |
| 19.9 | 34 | 20 | 5 |
| 44.3 | 36 | 27 | 8 |
| 54.15 | 22 | 16 | 3 |
| 54.31 | 32 | 25 | 7 |
| 71.5 | 4 | 5 | 2 |
| 87.3 | 63 | 44 | 15 |
| 99.5 | 16 | 13 | 3 |
| 104.3 | 17 | 18 | 5 |
| 116.1 | 8 | 4 | 1 |
| 116.3 | 17 | 12 | 3 |
| 2.6 | 21 | 17 | 8 |
| 2.7 | 9 | 7 | 2 |
| 54.20 | 33 | 29 | 9 |
| 59.6 | 54 | 37 | 14 |
| 60.6 | 13 | 11 | 4 |
| 5 | 49 | 39 | 16 |
| 20 | 37 | 22 | 9 |
| 62 | 7 | 5 | 2 |
| 63.1 | 75 | 58 | 20 |
| 69.1 | 21 | 17 | 2 |
| 69.5 | 20 | 15 | 5 |
| 81.5 | 26 | 12 | 3 |
| 91.2 | 43 | 39 | 19 |
| 108.4 | 55 | 45 | 16 |
| 109.3 | 27 | 28 | 8 |
| 109.4 | 69 | 59 | 22 |
| 123.2 | 34 | 35 | 10 |
| 123.3 | 31 | 20 | 5 |
| 123.7 | 79 | 68 | 16 |
| 7.2 | 21 | 17 | 4 |
| 7.3 | 24 | 20 | 6 |
| 7.4 | 7 | 8 | 2 |
| 6.2 | 19 | 12 | 4 |
| Totals | 1,079 | 994 | 323 |

The second experiment was the reciprocal of the previous one. A (mm) female, homozygous for spotted females and non-spotted

## Table IV.

### Back Cross Test

$P_1$: Mm heterozygous spotted female × mm homozygous non-spotted male.
$F_1$: Progeny:

| Pair No. | Females Spotted. | Males. | |
|---|---|---|---|
| | | Spotted. | Non-spotted. |
| | (Mm, mm) | (Mm) | (mm) |
| 4 | 23 | 13 | 10 |
| 71 | 31 | 19 | 20 |
| 86 | 32 | 16 | 17 |
| 96 | 10 | 4 | 3 |
| 105 | 0 | 1 | 2 |
| 119 | 22 | 7 | 7 |
| 125 | 21 | 17 | 16 |
| 3.1 | 26 | 13 | 10 |
| 3.2 | 32 | 9 | 10 |
| 3.5 | 10 | 7 | 6 |
| 3.6 | 30 | 16 | 10 |
| 17.3 | 12 | 7 | 4 |
| 198 | 18 | 11 | 15 |
| 51.4 | 3 | 2 | 3 |
| 51.6 | 36 | 18 | 13 |
| 54.1 | 20 | 22 | 23 |
| 54.2 | 45 | 20 | 12 |
| 54.4 | 25 | 14 | 13 |
| 54.14 | 7 | 4 | 3 |
| 54.16 | 37 | 18 | 18 |
| 54.30 | 28 | 10 | 9 |
| 60.1 | 131 | 47 | 69 |
| 60.3 | 31 | 11 | 19 |
| 60.4 | 27 | 14 | 14 |
| 60.8 | 43 | 22 | 23 |
| 60.9 | 30 | 13 | 19 |
| 85.3 | 34 | 20 | 16 |
| 86.2 | 74 | 40 | 52 |
| 87.1 | 126 | 68 | 50 |
| 94.4 | 24 | 7 | 11 |
| 94.5 | 13 | 4 | 6 |
| 98.2 | 31 | 5 | 6 |
| 98.3 | 29 | 10 | 12 |
| 99.1 | 23 | 17 | 10 |
| 99.2 | 103 | 34 | 34 |
| 99.3 | 4 | 3 | 3 |
| 99.4 | 96 | 44 | 42 |
| 119.1 | 11 | 7 | 9 |
| 125.3 | 37 | 18 | 11 |
| 127.1 | 52 | 24 | 24 |
| 128.4 | 11 | 3 | 4 |
| Totals | 1,375 | 679 | 658 |

males was bred to a (MM) male, homozygous for spotted males and females. The $F_1$ offspring from 24 separate pairs gave 488

heterozygous, spotted (Mm) females and 483 heterozygous, spotted (Mm) males. This test further shows the dominance of the spotted trait to the normal. Furthermore this experiment, when compared with the previous one, proves that the character is not sex-linked. Table II. gives the data.

The third test concerned heterozygous, spotted (Mm) females and heterozygous, spotted (Mm) males. The $F_2$ progeny from 43 $F_1$ single pairs totaled 1,079 spotted (MM, Mm, mM, mm) females, 994 spotted (MM, Mm, mM) males, and 323 non-spotted (mm) males. The result approaches a 4 : 3 : 1 sex-limited ratio, which shows that the spotted females appear identical, and that macula is dominant to normal. This data is tabulated in Table III.

The fourth experiment is a back cross with the $F_1$ hybrids, (Mm), heterozygous insects, and the normal type (mm) weevils. The parents were heterozygous, spotted (Mm) females and homozygous, non-spotted (mm) males. From 41 single pair matings 1,375 spotted (Mm, mm) females, 697 heterozygous, spotted (Mm) males, and 658 homozygous, non-spotted (mm) males, were obtained. The ratio, therefore, approximates a sex-limited 2 : 1 : 1. This result indicates further that normal spotting is recessive to macula. The data is listed in Table IV.

The fifth experiment was another back-cross test, the reciprocal of the previous one. Homozygous, normal, spotted (mm) females were mated with heterozygous spotted (Mm) males. From the 43 different pairs a total of 1,415 spotted (Mm, mm) females, 786 heterozygous spotted (Mm) males and 798 homozygous non-spotted (mm) males were obtained. This gave a 2 : 1 : 1 sex-limited ratio, or actually a 1 : 1 ratio, since the females appeared alike. Spotting is dominant to the normal type. Complete information is found in Table V.

In the sixth test heterozygous spotted (Mm) females and homozygous spotted (MM) males were used. The offspring from 26 separate pairs totaled 701 spotted (MM, Mm) females and 654 spotted (MM, Mm) males. This demonstrates the dominance of the macula character to its recessive normal trait, spotted females and non-spotted males. This data is compiled in Table 6.

## Table V.

Back Cross Test: Reciprocal Cross.

$P_1$: mm homozygous wild type female × Mm heterozygous spotted male.
$F_1$: Offspring:

| Pair No. | Females Spotted. | Males. | |
|---|---|---|---|
| | | Spotted. | Non-spotted. |
| | (Mm, mm) | (Mm) | (mm) |
| 6 | 33 | 30 | 27 |
| 6.1 | 2 | 4 | 4 |
| 31 | 27 | 20 | 15 |
| 91 | 33 | 18 | 19 |
| 108 | 20 | 8 | 9 |
| 100 | 24 | 10 | 13 |
| 103 | 41 | 24 | 19 |
| 1.2 | 34 | 14 | 15 |
| 20.7 | 16 | 8 | 9 |
| 31.6 | 31 | 19 | 26 |
| 34.3 | 16 | 9 | 6 |
| 34.2 | 11 | 3 | 4 |
| 39.1 | 25 | 16 | 19 |
| 78.1 | 31 | 33 | 38 |
| 78.3 | 17 | 4 | 4 |
| 79.1 | 29 | 11 | 17 |
| 79.3 | 48 | 19 | 19 |
| 79.7 | 13 | 6 | 4 |
| 81.4 | 33 | 15 | 18 |
| 88.2 | 62 | 33 | 33 |
| 88.7 | 15 | 7 | 11 |
| 91.1 | 31 | 16 | 14 |
| 91.3 | 95 | 37 | 34 |
| 91.6 | 34 | 60 | 51 |
| 92.1 | 15 | 7 | 12 |
| 92.5 | 73 | 22 | 28 |
| 92.6 | 157 | 83 | 78 |
| 108.1 | 5 | 7 | 12 |
| 123.1 | 67 | 28 | 29 |
| 123.4 | 40 | 19 | 16 |
| 64.1 | 6 | 7 | 5 |
| 64.2 | 8 | 10 | 11 |
| 112.1 | 33 | 13 | 16 |
| 112.2 | 64 | 37 | 31 |
| 9.10 | 42 | 19 | 23 |
| 54.11 | 3 | 2 | 3 |
| 54.28 | 30 | 13 | 17 |
| 82.2 | 25 | 15 | 11 |
| 116.5 | 29 | 12 | 10 |
| 128.1 | 27 | 23 | 17 |
| 10.10 | 8 | 4 | 5 |
| 101.2 | 15 | 10 | 10 |
| Totals | 1,415 | 786 | 798 |

The seventh experiment was the reciprocal of the previous one. The parents were homozygous, spotted (MM) females and

## Table VI.

Back Cross Test.

$P_1$: Heterozygous, Mm, spotted female × homozygous, MM, spotted male.
$F_1$: Offspring:

| Pair No. | Female Spotted. | Male Spotted. | Pair No. | Female Spotted. | Male Spotted. |
|---|---|---|---|---|---|
| | (MM, Mm) | (MM, Mm) | | (MM, Mm) | (MM, Mm) |
| 20.3...... | 1 | 1 | 47.5....... | 19 | 17 |
| 48.2...... | 4 | 5 | 49.1....... | 21 | 24 |
| 62.4...... | 17 | 15 | 49.2....... | 3 | 4 |
| 69.4...... | 9 | 4 | 47.7....... | 5 | 9 |
| 78.6...... | 70 | 56 | 47.8....... | 30 | 34 |
| 80.5...... | 3 | 3 | 48.1....... | 11 | 6 |
| 81.1...... | 33 | 16 | 68.5....... | 31 | 32 |
| 88.5...... | 3 | 1 | 68.6....... | 28 | 40 |
| 88.6...... | 22 | 5 | 68.7....... | 29 | 25 |
| 92.7...... | 131 | 149 | 79.5....... | 37 | 32 |
| 47.9...... | 37 | 25 | 80.2....... | 21 | 30 |
| 47.3...... | 43 | 34 | 80.4....... | 36 | 49 |
| 47.4...... | 28 | 20 | 81.8....... | 29 | 18 |
| | | | Totals.... | 701 | 654 |

heterozygous spotted (Mm) males. The offspring from 15 pairs, gave 236 spotted (MM, Mm) females and 213 spotted (MM, Mm) males. Hence, both sexes were homozygous for the macula (MM) genes, and heterozygous for the macula (M) gene and normal (m) factor. The macula mutation is dominant to the wild type. Complete data is presented in Table VII.

## Table VII.

Back Cross Test.

*Reciprocal Cross.*

$P_1$: Homozygous, MM, spotted female × heterozygous, Mm, spotted male.
$F_1$: Offspring:

| Pair No. | Female Spotted. | Male Spotted. | Pair No. | Female Spotted. | Male Spotted. |
|---|---|---|---|---|---|
| | (MM, Mm) | (MM, Mm) | | (MM, Mm) | (MM, Mm) |
| 17.1 ..... | 28 | 23 | 94.6 ...... | 10 | 5 |
| 17.2 ..... | 4 | 1 | 51.8 ...... | 6 | 9 |
| 51.2 ..... | 21 | 10 | 54.12...... | 3 | 0 |
| 51.3 ..... | 2 | 2 | 54.21...... | 36 | 33 |
| 51.11..... | 18 | 18 | 126.4 ...... | 9 | 17 |
| 59.2 ..... | 26 | 18 | 126.8 ...... | 18 | 18 |
| 59.3 ..... | 23 | 27 | 101. | 29 | 31 |
| 71.4 ..... | 3 | 1 | | | |
| | | | Totals..... | 236 | 213 |

The above series of experiments prove that the two elytra traits, normal and macula, differ in the male phenotypes only, since the factors for the macula (MM) mutation and the wild (mm) type appear identical for both female traits. These females are alike phenotypically but deviate genotypically, since the normal female has the same black four spotted pattern as its mutant, the macula female. Again, normal is recessive to macula.

Many sex-differences are detected in insects, occurring most frequently in Diptera; *Drosophila* probably furnishing the greatest number, with Lepidotera next, then Coleoptera. This sequence appears, as the result of the amount of genetic study directed upon these species. Many of the sex-limited traits in *Drosphila* are less distinct in one sex than in the other, thus differing somewhat from *Bruchus*, in which there is no visible manifestation of such characters in the male, except for the macula mutation.

Examples of these sex-limited traits for *Bruchus*, in which the male elytra is a non-spotted tan, have been previously demonstrated, hence they will be merely mentioned in this relation. The first mutations discovered (Breitenbecher, 1921) consisted of red, black, white, and tan elytra colors apparent only in the females. The respective male for each of these four female cultures was a non-spotted tan, the wild type elytra color. The four traits are multiple allelomorphs. A second non-visible trait in the male occurs with mosaic females (Breitenbecher, 1922). These females have elytra of different colors, often combined with varied spotting. In a third instance the males remained non-spotted tan, although the females displayed four red spots on the elytra (Breitenbecher, 1923). This character was dominant to the normal. Another mutation, in which the male showed a complete absence of the trait, was that in which all females were apterous. The males were fully winged. This was a recessive character (Breitenbecher, 1925). Lastly, another mutation, never visible in the male, was called "piebald." Here bilateral asymmetry was manifested since the females were of two types, about equal in numbers. One group had two red spots on the left elytron and two black spots on the right; the

other group had two black spots on the left elytron and two red ones on the right. When the two types of females were added, the trait was found to be inherited as a recessive to normal (Breitenbecher, 1925). Every male, for the entire list of characters enumerated, had non-spotted tan elytra similar to the wild type.

It is improbable that such latent characters in the male are caused by developmental differences, because in sex-limited, as well as other Bruchid mutations, the male emerges a fraction of a day before the female. Inhibitors associated with the male complex may produce this peculiarity. Or, since the female has two X-chromosomes, and the male only one X-chromosome, the sex-limited traits may be caused by pattern, or normal, genes within the X-chromosome. These may be associated with factors in other chromosomes in a degree enabling the character to be completely manifested in the female. The one sex-chromosome of the male may not be sufficient for complete manifestation of the trait. This conception is similar to that of Bridges (1922) for *Drosophila*.

The macula and normal patterns of *Bruchus*, illustrating sex differences, may be considered as the result of identical phenotypes for the female, since the females for each trait have duplicate spotting patterns on the elytra. The mm genes in the wild male are non-visible, while the MM factors for the normal four black spots are visible in the macula male.

It is to be hoped that some mutation may occur, in which the gene concerned with the entire pattern or non-pattern trait may appear. This might assure a definite solution for these sex-limited differences in *Bruchus*.

## Conclusions.

1. The macula mutation is dominant to the wild type.

2. Genes, mm, represent the wild type, having spotted females and non-spotted males.

3. Factors, MM, represent the macula mutation, in which both males and females are spotted.

4. The macula character is not sex-linked.

5. Inhibitors probably produce latent traits in the male.

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# THE EFFECT OF THYROID FEEDING ON THE MOULTING PROCESS AND FEATHER STRUCTURE OF THE DOMESTIC FOWL.

HARRY BEAL TORREY AND BENJAMIN HORNING.

I.

In the course of investigations on some of the endocrines and their functional correlations in the domestic fowl, it was observed that the addition of desiccated thyroid to the ration of Brown Leghorn and White Leghorn chicks led to striking modifications in the form of the first set of rectrices, and in the course of the first moult (Horning and Torrey, 1923). The form of the rectrices was modified conspicuously by the shortening of the vane and the lengthening of the naked region of the shaft. A characteristically "quilled" feather was the result, though somewhat reduced in total length. The course of the moult was modified by the maintenance of base-to-tip continuity between first and second rectrices, in sharp contrast to the normal independent and discontinuous development of these feathers.

The results of this experimentally produced hyperthyroidism, and others to be considered in another place, were then examined for the light they might throw upon the rôle of the thyroid hormone in feather differentiation, possibly in processes of differentiation in general. Feathers afford certain advantages as material, for they give permanent records of the progressive changes that occur in the feather germ during their development. But it is only of such a period that they do give reliable records, obviously enough, for once grown, they are removed from the influence of experimental procedures to which they might earlier have been susceptible.

2.

The following experiment is typical and with the accompanying tabulation will suffice to establish the fact that desiccated thyroid given in appropriate doses produces "quilling" of the rectrices of chicks of both sexes, with or without gonads.

Fifty-two Brown Leghorn chicks, hatched March 27, were divided into four groups as indicated in Table I.: two control groups (one with and one without gonads); two experimental groups (one with and one without gonads). A week later, on April 3, the rectrices of all were pushing out. On that date daily doses of 16 mgm. of desiccated thyroid (Armour's, containing 0.2 per cent. iodine) in capsule by mouth, were begun for one group of normal chicks (Nos. 36–50). On April 17, the dosage was increased to 32 mgm. and given to the second experimental group (Nos. 51–63) also.

TABLE I.

| Controls. | | | Thyroid-fed Chicks. | | |
|---|---|---|---|---|---|
| No. of Chick. | Sex. | Rectrices. | No. of Chick. | Sex. | Rectrices. |
| 25....... | Female | None quilled | 37...... | Male | 2 pairs quilled |
| 26....... | Female | " " | 38...... | Male | 4 " " |
| 27....... | Male | " " | 39...... | Female | 6 " " |
| 28....... | Female | " " | 40...... | Female | 4 " " |
| 29....... | Female | " " | 41...... | Female | 2 " " |
| 30....... | Male | " " | 42...... | Female | 5 " " |
| 31....... | Male | " " | 44...... | ? | 4 " " |
| 32....... | Male | " " | 45...... | Male | 4 " " |
| 33....... | Male | " " | 46...... | Female | 4 " " |
| 34....... | Female | " " | 47...... | Male | 3 " " |
| 35....... | Male | " " | 48...... | Male | 3 " " |
| 36....... | Female | " " | 49...... | Male | 2 " " |
| | | | 50...... | Fmeale | 5 " " |

| Gonadless Controls. | | | Gonadless Thyroid-fed Chicks. | | |
|---|---|---|---|---|---|
| No. of Chick. | Sex. | Rectrices. | No. of Chick. | Sex. | Rectrices. |
| 64....... | Male | None quilled | 51...... | Male | 3 pairs quilled |
| 65....... | Female | " " | 52...... | Male | 4 " " |
| 66....... | Female | " " | 53...... | Female | ? ? ? |
| 68....... | Female | " " | 54...... | Male | 6 pairs quilled |
| 69....... | Male | " " | 55...... | Male | 1 " " |
| 70....... | Male | " " | 56...... | Female | 2 " " |
| 71....... | Male | " " | 57...... | Male | ? ? ? |
| 72....... | Female | " " | 58...... | Female | ? ? ? |
| 73....... | Female | " " | 59...... | Male | ? ? ? |
| 74....... | Female | " " | 60...... | Male | 6 pairs quilled |
| 75....... | Male | " " | 61...... | Female | 1 " " |
| 76....... | Female | ? ? | 62...... | Female | 3 " " |
| 77....... | Male | None quilled | 63...... | Female | 1 " " |
| 78....... | Female | " " | | | |

The condition of the rectrices in all groups on May 18, 52 days after hatching, is shown in Table I.

The shortening of the vane characteristic of the "quilled" feathers was due to a failure of a considerable number of barbs to differentiate proximally. There was no sign of a loss of barbs previously formed and broken off. The vane of each "quilled" feather, though abnormally proportioned as already indicated, ended below in an aftershaft of barbules surrounding the superior umbilicus, with no sign that there had ever been barbs proximal to this point.

As is well known, the barbs of a feather arise from the so-called intermediate cells of the feather germ epidermis, these intermediate cells differentiating in the growing zone into parallel ridges, which, as the feather germ elongates, run a longitudinally oblique course about the dermal core. These ridges differentiate beginning with their distal ends into the barbs and barbules of the definitive feather and are continually renewed from below.

Now the effect of thyroid feeding was to abbreviate the period of ridge formation in the growing zone, and as a consequence to suppress prematurely in that region the processes of differentiation on which barb formation normally depends, without suppressing, however, the processes on which the lengthening of the calamus depends. It is true that the total length of each of the "quilled" feathers thus produced was somewhat less than normal, but that this was not to be attributed to a direct inhibitory effect of the thyroid upon the growth of the feather germ, but rather to a precocious development of the second rectrices, will appear, we believe, from facts to be given below. It may be said here, however, that while the suppression of barb ridge differentiation occurred prematurely after thyroid, in rare cases these ridges might appear again prematurely, after thyroid, about the inferior umbilicus of the first rectrix, foreshadowing the base-to-tip connection of first and second rectrices, to be described in the next section.

3.

In size, shape and coloration—details of which do not here concern us—the first rectrices, belonging to the first few weeks of the chick's life after hatching, differ distinctly from the second, which are adult in type.

FIG. 1. White Leghorn male about 8 weeks old, with normal rectrices On the extreme right is a rectrix of the first set; all the others are of the second set.

Normally the first or infant rectrices may drop away without urging from below; or they may be pushed out by the seconds that are developing from the same follicles. In neither case, however, are the two continuous. Though the succeeding

feather may arise from the same germinal papilla as its predecessor, a period of inactivity of the papilla follows the withdrawal from the calamus of the first and the rounding off of the inferior umbilicus. A new feather is then begun, within a sheath of its own.

The rounding off of the tip of the calamus of the first feather and the establishment of a discontinuity between the first feather and its successor involve certain processes of differentiation before the feather follicle and papilla become quiescent. One of the effects of thyroid feeding in our experiments was to suppress these terminal processes of differentiation by maintaining the papilla in continuous, even though, it may be, reduced activity. This continuity of process was revealed in continuity of structure —and, incidentally, settled, in this case, the question that has sometimes been raised whether successive feathers from the same follicle spring from the same papilla. Here the second obviously did spring from the same papilla as the first. If the feathers of succeeding moults arise similarly, the series, in place of a single feather, would appear to be due to a rhythmic variation in the velocity of critical reactions conditioning the development of the papilla. This variation, normally extreme and leading to discontinuity, is essentially independent of the normal thyroid secretion. The addition of thyroid to the ration under the conditions of our experiments lessened the amplitude of the rhythmic variation without determining the rhythm itself.

The "quilled" first rectrices were originally noticed in our thyroid-fed chicks as distal appendages to the members of the second set, since they were not being shed in normal fashion. Examination showed that the structure of the inferior umbilicus was unusual, the proximal end of the calamus being wide open and the plane of the orifice running ventrodorsally at a sharp angle with the axis of the calamus. Dorsally the lip of this oblique orifice was extended proximally as though the quill had been cut to form a long pen point.[1]

[1] In exceptional cases, as in a wing quill before us, this proximal extension is not a prolongation of the median dorsal line of the calamus, but of a region perhaps twenty degrees away from it. Though the median dorsal lines of the two feathers do not lie in the same plane, there is no obvious structural twist to account for the fact.

At the tip this penlike extension was continuous with the rhachis of the proximal feather. Elsewhere the lips of the orifice, including the edges of this dorsal extension, were continuous with the distal ends of about fifteen pairs of barbs of the proximal feather. The original orientation of the barbs, which had differentiated from long spiral ridges in the wall of the cylindrical feather germ, was thus preserved, and the orifice correspondingly obscured.

The falling of the distal feather was accomplished by rupture of these connections. As a result, the distal end of the second rectrix presented a frayed appearance. This came to be recognized as a characteristic of the second rectrices of thyroid-fed birds, in sharp contrast with the smooth rounded ends of the vanes of normal birds.

4.

What had been said regarding the effect of thyroid feeding on the development of the first rectrices and the first moult applies also to wing quills, though much less frequently and with slight unessential differences in detail; such, for instance, as the obliquity of the umbilical orifice. Contour feathers are not affected, according to our observations.

The facts may be briefly summarized. Thyroid feeding inhibits: (1) the differentiation of proximal barbs and barbules, thus shortening the vane without proportionately shortening the calamus; the proximal region of the vane passing into the after shaft normally without traumatic defect; (2) the usual differentiation of the inferior umbilicus; (3) the withdrawal of the feather pulp from the calamus, and the normal inactive period of the pulp. Continuity between first and second rectrices is the result.

Thyroid feeding also (4) accelerates the differentiation and eruption of the second rectrices. This acceleration was observed first in Rhode Island Red males (Torrey and Horning, 1922). That it occurs in Brown Leghorns is shown by the facts in the next paragraph. The precocious development of the second rectrices correspondingly limits the maximum growth period of the first and probably accounts, at least in part, for the reduced length of the latter after thyroid.

Thirty-two Brown Leghorn chicks hatched April 9 were divided into two lots, each containing 8 males and 8 females. One lot was fed desiccated thyroid, beginning April 14: 10 mgm. daily for 5 days, then 15 mgm. daily. On May 22, 43 days after hatching, the moult had proceeded as shown by the following table.

TABLE II.

| Controls. | | | | Thyroid-fed Chicks. | | | |
|---|---|---|---|---|---|---|---|
| No. of Chick. | Sex. | First Rect. | Second Rect. | No. of Chick. | Sex. | First Rect. | Second Rect. |
| 1.... | Female | 4 pairs | 3 pairs | 17...... | Female | 1 + 3 pairs | 4 pairs |
| 2.... | Female | 5 " | 1 " | 18...... | Female | 3 + 4 " | 4 " |
| 3.... | Female | 7 " | | 19...... | Female | 3 " | 3 " |
| 4.... | Female | 7 " | | 20...... | Female | 1 + 2 " | 4 " |
| 5.... | Female | 5 " | 2 " | 21...... | Female | 1 + 4 " | 4 " |
| 6.... | Female | 7 " | | 22...... | Female | 3 " | 4 " |
| 7.... | Female | 7 " | 1 " | 23...... | Female | 1 + 3 " | 4 " |
| 8.... | Female | 5 " | 2 " | 24...... | Female | 4 " | 3 " |
| 9.... | Male | 7 " | | 25...... | Male | 6 " | 1 " |
| 10.... | Male | 7 " | | 26...... | Male | 3 + 4 " | 3 " |
| 11.... | Male | 7 " | 1 " | 27...... | Male | 5 " | 2 " |
| 12.... | Male | 6 " | | 28...... | Male | 5 " | 2 " |
| 13.... | Male | 6 " | | 29...... | Male | 2 " | 4 " |
| 14.... | Male | 0 " | | 30...... | Male | 4 " | 3 " |
| 15.... | Male | 7 " | | 31...... | Male | died | |
| 16.... | Male | 7 " | | 32...... | Male | 3 pairs | 4 " |

The second rectrices recorded for the controls were just appearing. Those recorded for the thyroid-fed chicks were an inch or more longer. In chicks numbered 17, 20, 21 and 23 the variation in the notation indicates that one pair of first rectrices was "quilled" and continuous with the second; in number 26, 3 pairs were "quilled" and similarly continuous with the second.

5.

In certain respects the modifications in structure and moulting that we have described recall defects of structure and moult that have been associated with fault-barring through the work of Strong (1902), Riddle (1907, 1908, 1908*a*) and Whitman (1919).

Strong (1902) called attention to abnormal transverse bands in both remiges and rectrices of a hybrid pigeon, referable clearly to defective barbules. He also described and figured one of the body coverts, for the most part downy and carrying a

FIG. 2. White Leghorn male about 7 weeks old, that was fed thyroid from the age of two weeks. The outer pair of infant rectrices are not "quilled" and may be compared with their "quilled" mates intervening.

tassel-like distal appendage with a horny cylindrical base continuous with rhachis and barbs of the main body of the feather. The horn cylinder he interpreted as a region in which, during

the development of the feather, differentiation but not cornification had been omitted.

Riddle (1907) distinguished five types of defect in feather structure, two being represented by Strong's cases, a third by a very inconspicuous transverse depression or line without defects in barbules, a fourth by constrictions in the feather germ, and a fifth by a single instance of a feather with barbs broken off at even distances from the shaft, the defect extending parallel to the rhachis.

The first three of these are of widespread occurrence among birds. Riddle discussed them in connection with what Whitman had called fundamental bars (alternating lighter and darker transverse bands of pigment) so beautifully figured in his posthumous paper (1919, pls. 72–75). He concluded (1908) on the basis of experiments, that both are referable to variations in the nutrition of the feather germ during its development, the fundamental bars being produced by diurnal changes in blood supply, the fault bars by defective nutrition through this and other agencies. In his experiments, reducing the food, feeding Sudan III, mechanical destruction of tissues and blood vessels and the administration of amyl nitrite were all effective.

We have no satisfactory evidence of variation in the blood pressure of our birds. But we have seen abnormalities in feather structure belonging to Riddle's fifth type as a direct consequence of starving two White Leghorn males. One of our adult male Brown Leghorns on a daily ration of 1 gm. of dessicated thyroid replaced three pairs of remiges with feathers of normal length but abnormal in structure and color. The vanes were narrower and proximally misshapen as though fashioned under cramped conditions. The normal color was almost entirely lacking, the feathers being prevailingly white. There appears to be no reason to doubt that directly or indirectly such defects are referable to disturbed nutrition. We have also two rectrices from an adult Rhode Island Red female nearly two years old that resembles Strong's feather with a "tassel-like appendage." One of these rectrices is marked with great regularity by transverse depressions belonging to Riddle's third type of feather defect. At the distal end of the "appendage" two or three of these are definitive

fault bars, being represented by actual defects in barbule formation. Toward the base of this "appendage," as the vane narrows, they become more and more closely spaced. When the vane broadens out again, beyond this narrow neck, these lines are less closely but very regularly spaced. Assuming that the distance between successive lines is a measure of the daily growth of the feather, the setting off of distal appendage or lobe from the rest of the feather was correlated with a period of diminished growth velocity due to a cause not definitely known in this case, but very possibly a nutritional deficiency covering about fifteen days.

Riddle (1908*a*) interpreted in a similar way the relation between down and succeeding definitive feathers. Jones (1907) had shown that the first down and its succeeding definitive feather are produced by one continuous growth, the former being the plumulaceous tip of the latter. But the down feather is differentiated from the pennaceous feather, with which it is continuous, by a short quill, which Riddle (1908) regarded as a defective region due to the same general cause responsible for fault bars, namely, inadequacy of nutrition. True quills also, Riddle thought, resulted from a diminished food supply.

There are, then, certain points of resemblances between these phenomena which, whether normal or abnormal, have been interpreted as results of variations in the food supply, and the phenomena we have described as results of thyroid feeding. There are, however, certain significant points of difference.

Thyroid feeding shortens the vane of the first rectrix in chicks and increases the proportionate length of the calamus by the suppression of proximal barbs and their associated barbules. This resembles effects attributed to poor nutrition.

Thyroid feeding tends to maintain continuity between first and second generations of rectrices. We have no evidence that poor nutrition does this.

Thyroid feeding tends to accelerate the differentiation and eruption of the second rectrices. Poor nutrition, on the contrary, tends to retard these processes.

It appears then that these results of thyroid feeding are of two opposing sorts, only one of which is comparable with the

FIG. 3. Three wing feather from young Brown Leghorns showing continuity of feathers in succeeding generations as a result of thyroid feeding. Only a portion of each second generation feather is shown (below).

effects of nutritive deficiency. Such effects vary in intensity with the dose of thyroid administered. The "quilling" in the

birds recorded in Table I., more pronounced than in the birds of Table II., followed a heavier dosage of thyroid. And after the exceptionally large daily ration of 1 gram of dessicated thyroid one of our adult males already mentioned replaced 3 pairs of moulted remiges with feathers the proximal portions of whose vanes presented the deformities that characteristically follow starvation. There were pigmentation defects also, but these will be considered at another time.

The second consequence of thyroid feeding, namely, the acceleration of feather differentiation and eruption, would seem to involve an acceleration of division processes in the cells of the feather germ. According to Champy (1922) thyroid added for twenty-four hours to water containing frog tadpoles accelerates cell division in certain zones and tissues far beyond normal velocities. Ebeling (1924), using much more satisfactory methods, has established the accelerating effect on cell division of substances secreted by living thyroid which is added to cultures of fibroblasts *in vitro*. Whether thyroxin would act similarly is not yet known. Thyroxin (Squibb's) does not accelerate the division rate of *Paramecium*, but, on the contrary, in dilutions of 1 : 1,000,000 actually depresses it (Riddle and Torrey, 1923; Torrey, 1923, 1924; Torrey, Riddle and Brodie, 1925). The evidence from our present material will be offered at a later date.

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March 12, 1924.

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# ON THE ACTION OF CERTAIN SUBSTANCES ON OXYGEN CONSUMPTION.

## VI. The Action of Acids.

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In recent years the rôle of acidity and alkalinity in biological processes has been the subject of numerous investigations. The impetus to this field of research was given by the invention of methods for determining the true hydrogen ion concentration of biological fluids and materials. As a result of the great mass of work accumulating along this line of research, there prevails among biologists the impression or opinion that hydrogen ion concentration is of tremendous importance in the life of organisms. Yet, looking back upon the history of science, one may be pardoned for a certain degree of skepticism. Biologists appear prone to attach undue significance to whatever field of investigation happens to be the fashion of the decade and time alone can assign a mass of research on one particular topic to its proper place.

The present series of experiments was undertaken in part as a test of the proposition that hydrogen ion concentration is of fundamental importance in physiological processes. The consumption of oxygen in respiration was chosen as a physiological process essential to the organism in the highest degree and readily susceptible to quantitative measurement. An attempt was made to determine: (*a*) whether increasing the acidity of the environment of an organism has any effect upon the rate of oxygen consumption; (*b*) whether this effect is assignable to the free hydrogen ions or to some other factor. The experiments prove that alterations in external acidity markedly affect the rate of respiratory metabolism of animals; but they also show that the free hydrogen ions are little, if at all, responsible for the observed effect.

## Literature.

Surprisingly enough, very few investigations have been carried out on the effect of increased acidity on the respiratory metabolism of organisms. A rather careful search through the iterature has revealed only the following researches.

Among plants, only the lower forms have been investigated. Brooks ('21, '22) measured the carbon dioxide production of *Bacillus butyricus*, *B. subtilis*, and *B. tuberculosis* at various hydrogen ion concentrations. The bacteria were tested in dextrose solutions made in distilled water and the desired acidity was obtained with sulphuric acid. The rate of carbon dioxide production was at the maximum at pH 7.0 for *B. butyricus*, at pH 6.8 for *B. subtilis*, and was decreased at all acidities greater than these. In the case of *B. tuberculosis*, however, the carbon dioxide production was constant at all acidities between pH 7.4 and 4.4 and was decreased only at acidities greater than 4.4. Gustafson ('20) studied the effect of altered acidity on the respiration of the mold *Penicillium chrysogenum*. Variations in acidity between pH 8.0 and pH 4.0 were without effect upon the rate of carbon dioxide production. In acidities ranging from pH 4.0 to 2.65, the carbon dioxide output first rose and then fell; below 1.95 an irreversible decrease in the output was observed. The oxygen consumption was measured at pH 2.0 and was found to be markedly increased over the normal, thus agreeing with the initial carbon dioxide production at this particular acidity. In Gustafson's experiments, the mold was tested in dextrose solutions in distilled water and sulphuric and phosphoric acids were employed to increase the acidity.

Among animals, a few researches are available on eggs, tissues, and intact animals. Loeb and Wasteneys ('11) found that the oxygen consumption of sea-urchin eggs is slightly decreased in sea-water acidified to pH 6.0 to 4.0, and considerably decreased at or below pH 4.0. Thunberg ('09, '10) has carried out a large number of experiments on the action of acids on the rate of oxygen consumption and carbon dioxide production of surviving frog's muscle. The tests were performed in salt solution. Thunberg unfortunately did not determine the pH of his solutions—this procedure was not customary at that time—but gives the

concentration of acid in terms of molar strength. Hydrochloric acid decreased both oxygen consumption and carbon dioxide output of the muscle markedly, the decrease varying from 15 per cent. in 1/200 mol. HCl to 72 per cent. in 1/20 mol. HCl. Thunberg also tested the action on gaseous exchange of the frog's muscle of a large number of organic acids, including mono-, di-, tri-, and oxycarboxylic acids of both the paraffin and olefin series. Each acid was tested in the following concentrations: 1/100, 1/25, 1/10, and 1/5 molecular strengths. In the majority of the acids, both the oxygen consumption and carbon dioxide production were decreased at all concentrations but not very greatly so. The maximum decrease with the strongest concentrations was 15–25 per cent. In some of the acids (*e.g.*, propionic, butyric, lactic), the respiratory rate was increased in the more dilute concentrations, decreased in the stronger solutions. In fumaric, malic, and citric acids, the carbon dioxide output was greatly increased, while the oxygen consumption was but little altered. On the other hand, oxalic and malonic acids were found to decrease the carbon dioxide output to a much greater extent than the oxygen consumption. Succinic acid alone decreased the former while increasing the latter. It must be remarked that the concentrations used by Thunberg are extremely high and it is difficult for me to believe that the muscle could have remained uninjured in any of the solutions employed by Thunberg, even though each experiment lasted for only thirty minutes. Exposure to 1/50 or 1/20 mol. HCl, for instance, concentrations used by Thunberg, is, I believe, rapidly fatal to any living tissue. Thunberg does not consider the possibility of injury to the muscle in his experiments and makes no statements concerning the condition of the muscle during or after the exposure. I do not believe his experiments can be accepted as of physiological significance. Gray ('24) has studied the action of acids on the oxygen consumption and activity of ciliated tissues of bivalves. Acetic, butyric, and hydrochloric acids were used, presumably in sea-water. In the acidified water, both ciliary activity and oxygen consumption were diminished and in relation to the amount of acidity. Acetic acid at a pH of 4.6 to 4.2 and butyric acid at 4.2 caused a very great decrease and even complete

abolition of oxygen consumption. Hydrochloric acid was less effective than either of the two organic acids, producing only 50 per cent. depression at pH 3.0.

There is a dearth of experiments on entire animals. Jewell ('20) tested the carbon dioxide output of frog tadpoles in distilled water acidified with HCl. The output was decreased to 92 per cent. of the normal at pH 7.0, to 84 per cent. at pH 5.4, to 53 per cent. at 3.8, and to 37 per cent. at 3.2. Powers ('23) studied the effect of carbon dioxide tension on the oxygen consumption of the herring. He found the maximum oxygen consumption at pH 7.6 to 7.8; alterations in the carbon dioxide tension, and hence pH, either above or below this value, lead to a decrease, rather slight, however, in the amount of oxygen consumed by the fish. Powers ('22) is of the opinion that the ability of fish to utilize oxygen depends to some extent on the pH of the water in which they are living.

In addition to such direct measurements of the effect of increased acidity of the medium on respiratory exchange there is available a considerable number of researches on the action of acids on processes in which respiratory metabolism is undoubtedly of great importance. Acidification of the medium has a general retarding or depressing effect on such processes. I can refer here only briefly to this literature. It is a well known fact, attested by many researches, that acidification of the medium retards the cleavage and development of the egg and produces abnormal types of larvæ (cf. Loeb, '98, Moore, Roaf, and Whitley, '05, Child, '16, Medes, '17, and Smith and Clowes, '24, for echinoderm eggs; Child, '25, for hydroids; Child, '17, for annelids; Loeb, '98, and Hall, quoted by Shelford, '23, for teleost eggs; and Hall, '24, and Bellamy, '19 and '22, on amphibian development). The retarding effect of acids on regeneration was noted by Jewell ('20). Acidification of the medium constitutes a general method for altering and controlling polarities (cf. Child, '23, and Rustia, '25).

For a general summary of the rôle of acids in the behavior and life of aquatic organisms the excellent paper of Shelford ('23) should be consulted. I am unable however to agree with Shelford that hydrogen ion concentration is of greater importance

than carbon dioxide content for aquatic organisms. The facts that Jewell ('22) found an extensive and varied fauna in an acid stream (pH 5.8 to 7.1) and Jewell and Brown ('24), several fishes living in an acid lake (pH 4.4) indicate that hydrogen ion concentration is not of paramount importance in the distribution of aquatic animals. Allee ('23) noted a lack of relation of hydrogen ion concentration to the distribution of marine animals.

From the investigations cited the general conclusion is certainly justifiable that the acidification of the medium to the proper extent retards or depresses biological processes and activities. My own experiments are in agreement with this general result. But without further analysis the conclusion should not be drawn that the observed effect is assignable to the free hydrogen ions of the acidified medium. Natural waters usually contain salts which undergo chemical changes when the water is acidified. In particular, carbon dioxide is evolved. Unless it is definitely proved that the evolution of carbon dioxide or other secondary chemical changes attendant on acidification of natural waters is not concerned in the result, the observed effect should not be assigned to the hydrogen ion concentration *per se.* Even ordinary distilled water contains some carbonates and other salts which may affect the result. Some of the researches cited in the foregoing review cannot be criticized on this score, or only to a slight extent, as distilled water was used, but in other cases, where natural waters were employed, the carbon dioxide evolved was probably of more consequence in the result observed than the hydrogen ion concentration.

In an extensive study of the depressing effect of acidification of natural waters on the oxygen consumption of aquatic animals, presented in this paper, it is shown that in all probability, carbon dioxide is chiefly responsible for the effect.

## Methods.

The general course of procedure in the experiments to be reported was as follows. The rate of oxygen consumption of the animals in normal water was determined for a definite period of time, generally one hour. The normal water was then acidified to the desired extent and the oxygen consumption in

this acidified water for one or more successive periods of time determined. The animals were placed in Erlenmeyer flasks, if small, or in wide-mouthed salt bottles, if large, of about 500 cc. capacity. The water to be used was thoroughly aërated and placed in a large elevated receptacle. From this it was siphoned into the flasks or bottles containing the animals and allowed to flow out at the top for a few minutes. The flasks or bottles were then tightly closed and kept in a water bath at constant temperature for the desired length of time, generally one hour. After thoroughly shaking the contents, a sample of 125–150 cc. was then drawn by siphon and analyzed for oxygen content by Winkler's method. Blanks of the water used were of course also drawn at the beginning of the experiment and allowed to stand in the water bath with the animals until the end of the test. The difference between the oxygen content of these blanks and the samples drawn from the flasks or bottles containing the animals gives, after suitable calculation, the amount of oxygen consumed by the animals in the time selected. Immediately after drawing the normal samples, the acidified water was added in the same way and the animals allowed to respire in the acidified water for the same length of time. This was repeated as many times as desired, using the same animals and the same degree of acidification in any one series of experiments. The water was freshly acidified and aërated for each of these determinations. There is no possibility that alterations in the oxygen content of the water in any way affected the result, as the oxygen content was at saturation at the beginning of each test, and the test continued only long enough for the animals to use up a small part of the oxygen present.

The hydrogen ion concentration was determined by means of indicators purchased from the La Motte Chemical Products Company. A set of standard tubes covering the range from pH 3.0 to pH 8.4, with indicators brom phenol blue, brom cresol green, brom cresol purple, and phenol red, was employed. Solutions of these indicators were obtained from the same company. A few drops of the appropriate indicator added to 10 cc. of the water to be tested yields a color which can be compared with that of the standard tubes; the approximate pH is thus

easily obtained. Brom cresol green was not found to be reliable and consequently pH 5.0 could not be determined very exactly.

The acids employed were: hydrochloric, nitric, sulphuric, carbonic, acetic, butryic, citric, and tartaric. Each of these acids was used in the following concentrations, in terms of pH: 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, and in some cases 4.5. At least three experiments at each concentration of each acid were performed and in many cases a greater number of trials was deemed necessary. It will be seen that the amount of labor involved was very great and this fact must serve as my excuse for any deficiencies in the investigation which may suggest themselves to the reader.

## Experiments with Marine Animals.

The experiments recorded in this section were performed in 1922 at the Hopkins Marine Station, Pacific Grove, California. The animals used were two species of starfish, *Leptasterias equalis*, and *Patiria miniata*, and the nudibranch, *Anisodoris nobilis*. It may be noted in passing that starfish and nudibranchs have been found to constitute very favorable material for respiratory experiments.

The animals were placed in wide-mouthed salt bottles and the tests carried out as outlined above. *Leptasterias* is a small species and several individuals were therefore used in each bottle. As the other two species are larger, generally only one individual was necessary. Generally two successive trials of the oxygen consumption in normal sea-water were carried out. The sea-water was then acidified to the desired amount with hydrochloric acid and a determination immediately carried out. The animals were then usually allowed to stand for two to several hours in the acidified sea-water; another test was then performed. The animals then remained in the acid water over night, about 13 to 15 hours in different experiments, whereupon a third test of the respiration in the acidified water was performed. The animals were then returned to normal sea-water, and after several hours the oxygen consumption was again tested in normal sea-water.

It should be understood that for each test freshly aërated and freshly acidified water was used. The water was brought in directly from the ocean as the sea-water coming through the

pipes into the laboratory was found to be toxic. In acidifying the water, concentrated HCl was regarded as a 10 mol. solution and sufficient of this was added to a measured quantity of sea-water to give theoretically a certain molecular concentration of hydrochloric acid. Actually, of course, owing to the salts in the sea-water, the concentration obtained is less than that given in the tables. The hydrogen ion concentrations of these solutions could not be determined as I did not have with me the necessary outfit. It may be roughly estimated, however, that 1/1000 HCl in sea-water is about pH 7.0, 1/800 about pH 6.5, 1/600 about pH 6.0, and 1/400 between 5.0 and 5.5. When allowed to stand, of course, such solutions become continuously more alkaline, owing to the escape of carbon dioxide. For this reason, the solution was not made until a few minutes before it was to be used in the tests. The pH of normal sea-water is about 8.2.

The acidification of the sea-water has little or no noticeable effect upon the behavior of the animals in the lower concentrations, 1/1000 mol. or less. In concentrations of 1/800 mol. or greater, the animals are markedly affected. The starfish withdraw their tube feet into the ambulacral groove. The rays are retroflexed towards the aboral surface. In this position the animals remain during the exposure to acid and are apparently unable to move about or to cling with the tube feet. Nudibranchs display a similar behavior. In the stronger solutions they lose their ability to hold to the substratum with the foot and float about in the water in a state of immobility. The gills however remain expanded and it is not believed that differences in the degree of expansion of these organs are to any extent responsible for the depressing effect of acid on the oxygen consumption. In brief, acidification of the water appears to paralyze the neuro-muscular apparatus and to reduce the animal to a state of forced immobility. Recovery is prompt and complete upon return to normal sea-water. None of the concentrations used had any injurious effects upon the animals.

The results of the experiments are given in part in Tables I and II. A typical experiment for each concentration of acid used for one of the starfish species (*Patiria miniata*) and for the nudibranch is presented in Table I. Three trials of each concen-

## Table I.

Effect of the Acidification of the Sea-water with Hydrochloric Acid on the Rate of Oxygen Consumption of the Starfish *Patiria miniata*, and the Nudibranch *Anisodoris nobilis*.

Oxygen consumed given in cubic centimeters, per ninety minutes for the starfish, per hour for the nudibranch. Each vertical column constitutes one experiment, showing successive tests of the oxygen consumption of the same animals or animal. The concentration of the acid is given in molecular strengths.

| Starfish. | | | | Nudibranch. | | | |
|---|---|---|---|---|---|---|---|
| Oxygen Consumption in Normal Sea-water. | | | | | | | |
| .43 | .40 | .31 | .34 | .78 | 1.02 | .68 | .83 |
| Oxygen Consumption in Sea-water Acidified with HCl. | | | | | | | |
| 1/1000 | 1/800 | 1/600 | 1/400 | 1/1500 | 1/1000 | 1/800 | 1/600 |
| Immediate Effect. | | | | | | | |
| .41 | .32 | .23 | .22 | .76 | .77 | .49 | .50 |
| Effect after 2 to 6 Hours' Exposure. | | | | | | | |
| .35 | .31 | .21 | .22 | .74 | .78 | .57 | |
| Effect after 13 to 15 Hours' Exposure. | | | | | | | |
| .42 | .34 | | | .74 | .75 | | |
| Recovery on Return to Normal Sea-water. | | | | | | | |
| .42 | .44 | .32 | .34 | .76 | .97 | .72 | |
| Initial per cent. Depression. | | | | | | | |
| 5 | 20 | 26 | 36 | 3 | 25 | 28 | 40 |

## Table II.

Average Initial per cent. Depression of All Experiments on Marine Animals.

| Conc. HCl. | 1/1500 | 1/1000 | 1/800 | 1/600 | 1/400 |
|---|---|---|---|---|---|
| *Leptasterias* | | 31 | 38 | 38 | 51 |
| *Patiria* | | 9 | 21 | 28 | 34 |
| *Anisodoris* | 7 | 30 | 26 | 31 | |

tration were made but only one of these is given in the table, and may be taken as typical of the three. The results on the other species of starfish (*Leptasterias equalis*) being in no wise different from those on Patiria are omitted for the sake of economy of space. In Table II. is given a summary of all of the experiments, the three trials with each concentration being

averaged. The percentage of depression is figured from the oxygen consumption during the first exposure to the acid.

These experiments, although somewhat preliminary in nature, nevertheless exemplify the action of acids on respiratory metabolism. The conclusions which they furnish were verified by later more extensive work. These conclusions may be briefly stated at this point:

1. Acidification of the medium lowers the rate of oxygen consumption of animals.

2. This depression is greater the greater the degree of acidification up to a certain point.

3. Low concentrations are, however, relatively more effective than high concentrations.

4. After a certain degree of acidification has been attained, further acidification does not greatly increase the amount of depression of the oxygen consumption. The maximum depression obtainable is about 50 per cent.

5. The depressing effect of acids on respiratory metabolism is completely reversible, providing the concentration of acid employed is not actually injurious to the tissues of the animal.

## Experiments with *Planaria* in Unaltered Fresh Water.

The experiments to be reported in the remainder of the paper were performed at Chicago and occupied the greater part of my time from October, 1923, to February, 1925. The aim of the experiments was to test the effect upon the oxygen consumption of a fresh water animal of the acidification of fresh water with various acids at a variety of hydrogen ion concentrations. The animal selected for this purpose was the flatworm, *Planaria dorotocephala*. It was chosen for a variety of reasons: it is very abundant, is easily kept under laboratory conditions, has no calcareous parts which might be affected by acid, can be kept quiet during respiratory work, and finally had already served as material for a large amount of physiological experiment. I had originally intended to work also with fish and crayfish but the experiments on *Planaria* required a much greater expenditure of time than I had anticipated and I did not feel justified in spending any more time on the matter. I was further informed

by Dr. E. B. Powers that he was already engaged in a similar investigation on fish, and I was quite willing to relinquish this particular task to him.

1. *General Procedure.*—There is little to add to the procedure already outlined. Large stocks of *Planaria dorotocephala* are always at hand in our laboratory. Only stocks which had sojourned in the laboratory for at least one or two months were employed, as the basal respiratory rate of freshly collected material is variable. Only worms which had starved from four days to two weeks were used, since during this period the respiratory rate is constant. Each lot of worms to be used for an experiment consisted of a number of individuals sufficient to consume a readily measurable amount of oxygen in an hour. Such a lot was placed in a 500 cc. Erlenmeyer flask at least several hours before the experiment because when the worms are placed in any clean strange container they are apt to travel about restlessly for some time. By placing the worms in advance in the container in which they are to be tested and by darkening them during the experiment, movement can be practically entirely eliminated. It is certain that movement does not play any rôle in the results. Three such lots of worms were carried throughout the work in order that three tests of each strength of each acid could be performed simultaneously. This procedure shortens considerably the amount of labor involved. Each lot of worms was kept in a particular flask for nearly two weeks and used several times during that period. If desired such lots could then be fed once or twice in the flasks and after four days again utilized for experiments. This procedure was found most suitable because different lots of worms are apt to yield different percentages of depression with the same concentration of the same acid. To obtain a graded set of results showing a graded increase in the percentage of depression with increasing concentration of acid, it is almost necessary to test all of the concentrations of any one acid on the same lot of worms. If different lots are interpolated in such a series, the relation between depression and concentration is less regular, although always exhibiting the same general trend.

The remainder of the procedure was as already described. It

may be repeated once more that for each hour's test freshly aërated water was employed and this was freshly acidified to the desired degree a few minutes before being used for each hour's determination. Each test lasted for one hour. A determination of the normal rate of oxygen consumption in normal water (pH 8.0) was first run, and this was followed without pause by a determination in the acidified water. In many cases there were two or three trials in the acidified water, usually with intervals between the trials. During such intervals the animals remained of course in the acidified water. As it was found by repeated experiment that the maximum percentage of depression is obtained on the initial exposure to the acid, these later tests in acid were omitted in the latter part of the investigation. Generally the investigation of each acid was begun with the lowest concentration (pH 7.5) and the concentration increased by steps of 0.5 pH at each succeeding experiment. As will be discussed later this procedure gives the most regular results and great irregularities are introduced if the strongest concentrations are employed first.

There is not the slightest possibility that any differences in the oxygen content of the water either at the start of each test or produced during the test by the withdrawal from the water of oxygen by the animals in any way affects the results. The oxygen consumption of *Planaria dorotocephala* is entirely independent of the oxygen content of the water at all concentrations between 8 and 2 cc. per liter. Whether it is affected by concentrations above or below these limits has not been determined. Suffice it to say that in none of the experiments here recorded nor in any that I have reported in the past with this species has the oxygen content of the water ever reached a value which could have affected to the slightest degree the rate of oxygen consumption of the animals.

The acids used and the hydrogen ion concentrations at which they were tested have already been stated. Concentrations which might have injured the animals during the exposure were avoided. For this reason very few tests were run at acidities greater than pH 5.0. It was desired to keep within physiological concentrations.

All of the experiments on *Planaria* were performed at a temperature of 20° C. and the oxygen in all cases is calculated for this temperature. The results are given in cubic centimeters of oxygen consumed per hour. The animals were not weighed as the oxygen consumed per gram is of no importance for the present experiments. For the benefit of those who may be curious regarding this matter it may be stated that the normal rate of oxygen consumption of this species under the conditions of the experiments (worms of medium size, four to fourteen days after feeding, temperature 20° C.) is about .20 cc. of oxygen per gram per hour.

2. *Water*.—The water used in the experiments comes from a well driven into the ground from the floor of the basement of the laboratory. This water when it emerges from the taps is supersaturated with air and also contains a considerable amount of free carbon dioxide. It has a pH of about 7.3. On account of the gas content the water is heated but not boiled and then allowed to cool overnight before being used for experimental purposes. Water so treated has a pH of 8.0 to 8.2. This is the water regarded in the experiments as "normal" and the respiration in such water is taken as "normal." The water is of course thoroughly aërated before use, bringing the oxygen content up to 6 to 8 cc. per liter.

TABLE III.

ANALYSIS OF THE WATER USED IN THE EXPERIMENTS ON *Planaria*.

All figures represent parts per million. Analysis made in 1922.

| Determinations as | | Hypothetical combinations. | |
|---|---|---|---|
| Iron—Fe | 0.2 | Potassium nitrate | 1.4 |
| Manganese—Mn | 0.1 | Potassium chloride | 1.1 |
| Silica—$SiO_2$ | 7.8 | Sodium chloride | 7.6 |
| Nonvolatile | 0.7 | Ammonium chloride | 0.1 |
| Alumina—$Al_2O_3$ | 1.6 | Magnesium chloride | 1.0 |
| Calcium—Ca | 49.2 | Magnesium sulphate | 22.1 |
| Magnesium—Mg | 16.7 | Magnesium carbonate | 41.5 |
| Ammonia—$NH_4$ | 0.03 | Calcium carbonate | 123.0 |
| Sodium—Na | 3.0 | Silica—$SiO_2$ | 7.8 |
| Potassium—K | 1.1 | Alumina—$Al_2O_3$ | 1.6 |
| Sulphate—$SO_4$ | 17.7 | Iron oxide—$Fe_2O_3$ | 0.3 |
| Nitrate—$NO_3$ | 0.9 | | |
| Chloride—Cl | 6.0 | | |
| Alkalinity (methyl orange) | 170 | | |
| Residue | 193 | | |

The water used is thus the ground water of the Chicago region. It is high in salt content, particularly in carbonates. This water has been analyzed for us by the State Water Survey Division of Illinois. It seems desirable to present the analysis at this point.

3. *Behavior of Planaria in Acid.*—When the water is acidified no alteration in the behavior of the worms is noticeable until a certain degree of acidification is reached. This degree is different for different acids, but is generally in the neighborhood of pH 5.5 to 5.0. The animals lose the ability to glide about, owing apparently to a paralysis of the ciliary mechanism. They exhibit continuous writhing movements, and secrete a large amount of mucus. With greater acidification, they lose ability to cling to the glass and fall to the bottom of the container, where they remain, often with slight writhing movements. They are, when this condition is attained, extremely elongated and generally more or less curved, the ventral surface being concave, the dorsal convex. In short, as in the case of the marine animals, a sufficient degree of acidity induces a paralysis of the motor mechanism, which appears to involve the cilia first, the muscles later.

4. *Experiments with Hydrochloric Acid.*—A large number of experiments, about seventy, were performed with this acid. The normal respiration in normal water (pH 8.0) for one hour was first determined. The water was then acidified to the desired degree and the rate of oxygen consumption in the acid water immediately tested. An interval of one hour in the acidified water was then passed, another determination made in freshly acidified water, another hour passed, and a third determination carried out. It will thus be evident that the respiration during the first, third, and fifth hours in the acid was tested. This was the general procedure in the earlier part of the work. The acidity ranged from pH 7.5 to 4.5 at 0.5 pH intervals. It seems unnecessary to present in detail the mass of data thus accumulated as all of the experiments gave the same general result. A set of typical determinations of the action of hydrochloric acid at different hydrogen ion concentrations is given in Table IV. The averages of all of the experiments performed with this acid are summarized in Table VI. The action is graphed in Fig. 1.

## Table IV.

Action of Hydrochloric and Sulphuric Acids at Different Hydrogen Ion Concentrations on the Oxygen Consumption of *Planaria*.

All figures represent cc. of oxygen consumed per hour at 20° C. Each vertical column constitutes one experiment, giving successive determinations on the same lot of worms.

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal Respiration, pH 8.0. | | | | | | | | | | | | | |
| | .42 | .44 | .37 | .48 | .34 | .34 | .42 | .43 | .38 | .40 | .50 | .58 | .43 |
| Respiration in Acidified Water. | | | | | | | | | | | | | |
| | Hydrochloric. | | | | | | | Sulphuric. | | | | | |
| pH.......... | 7.5 | 7.0 | 6.5 | 6.0 | 5.5 | 5.0 | 4.5 | 7.5 | 7.0 | 6.5 | 6.0 | 5.5 | 5.0 |
| 1st hr......... | .36 | .34 | .28 | .34 | .23 | .20 | .23 | .36 | .27 | .27 | .33 | .37 | .24 |
| 3d hr......... | .38 | .37 | .33 | .37 | .24 | .22 | .25 | .38 | .30 | .34 | .39 | | |
| 5th hr........ | .38 | .33 | .30 | .38 | .27 | .20 | | .34 | .34 | .26 | .43 | | |
| Per cent. Initial Depression. | | | | | | | | | | | | | |
| | 13 | 23 | 25 | 29 | 33 | 40 | 46 | 16 | 29 | 35 | 34 | 37 | 45 |
| Recovery after Return to Normal Water. | | | | | | | | | | | | | |
| | .44 | .41 | .41 | .41 | .34 | .32 | .35 | .49 | .39 | .41 | .44 | .49 | .41 |

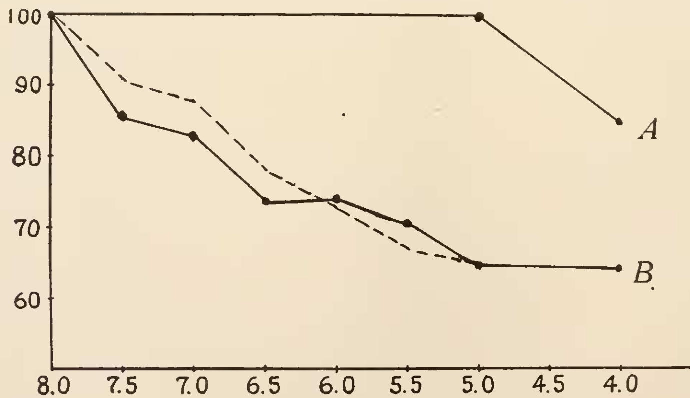


Fig. 1. Graph of the action of acids on the rate of oxygen consumption. Curve *A*, action of hydrochloric acid in carbonate-free water. Curve *B*, action of hydrochloric acid in ordinary water. Dashed line, average curve of the depressing action, obtained by averaging all of the acids except butyric. Percentage of depression, normal respiration being taken as 100, on the ordinate, pH on the abscissa.

It will be perceived by examining these tables that the rate of oxygen consumption of *Planaria* is decreased by acidifying the water with hydrochloric acid and that the percentage of depression is greater the higher the acidity. Yet it must be remarked at this point that the percentage of depression obtained with any given concentration of acid is subject to inexplicable variation even on the same lot of worms and quite commonly when different lots of worms are employed. In most cases this variation did not exceed 10 per cent. but it may reach 20 to 30 per cent., in the higher concentrations. On account of these puzzling variations I was frequently compelled to repeat the experiments many more times than had been anticipated as necessary. The matter of variation is considered at more length later and a possible cause is suggested.

### Table V.

Action of Carbonic and Acetic Acids at Different Hydrogen Ion Concentrations on the Oxygen Consumption of *Planaria*.

All figures represent cc. of oxygen consumed per hour at 20° C. Each vertical column constitutes one experiment, giving successive determinations on the same lot of worms.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal Respiration, pH 8.0. | | | | | | | | | | | | |
| | .34 | .21 | .31 | .29 | .28 | .25 | .40 | .50 | .50 | .36 | .51 | .71 |
| Respiration in Acidified Water. | | | | | | | | | | | | |
| | Acetic Acid. | | | | | | Carbonic Acid. | | | | | |
| pH | 7.5 | 7.0 | 6.5 | 6.0 | 5.5 | 5.0 | 7.5 | 7.0 | 6.5 | 6.0 | 5.5 | 5.2 |
| 1st hr. | .30 | .26 | .28 | .24 | .20 | .14 | .44 | .46 | .39 | .27 | .35 | .43 |
| 3d hr. | .36 | .26 | .37 | .28 | .25 | .14 | | .45 | | .25 | | |
| 5th hr. | .31 | | .32 | | | | | | | | | |
| Per cent. Initial Depression. | | | | | | | | | | | | |
| | 12 | +23 | 10 | 18 | 29 | 44 | +10 | 8 | 22 | 25 | 32 | 40 |
| Recovery after Return to Normal Water. | | | | | | | | | | | | |
| | .36 | .20 | .29 | .28 | .31 | | .41 | .50 | .46 | .44 | .52 | |

5. *Experiments with Sulphuric and Nitric Acids.*—The action of these two mineral acids was very similar to that of hydrochloric acid. The percentage of depression induced was consistently slightly greater at the same hydrogen ion concentrations

than that produced by HCl. Some detailed data on sulphuric acid are given in Table IV. and the results of all experiments with nitric and sulphuric acids are summarized in the general table, Table VI.

Table VI.

Summary of All Experiments with All Acids, giving the Average Percentage of Depression on Initial Exposure to Each Acid at Each pH Used.

| pH. | 7.5 | 7.0 | 6.5 | 6.0 | 5.5 | 5.0 | 4.5 | 3.8 |
|---|---|---|---|---|---|---|---|---|
| Hydrochloric | 14 | 17 | 26 | 26 | 29 | 35 | 45 | 36 |
| Sulphuric | 21 | 22 | 27 | 33 | 33 | 34 | | |
| Nitric | 18 | 26 | 28 | 30 | 40 | 40 | | |
| Carbonic | + 2 | 3 | 20 | 21 | 31 | 45 | | |
| Acetic | 6 | + 8 | 10 | 31 | 30 | 40[1] | | |
| Butyric | 16 | 7 | 15 | 7 | 8 | 37[1] | | |
| Tartaric | 13 | 14 | 22 | 21 | 31 | 33 | | |
| Citric | + 4 | 10 | 18 | 31 | 40 | 29 | | |

6. *Experiments with Carbonic Acid.*—Carbon dioxide gas was passed through the water until the desired acidity was obtained. In this procedure great care was necessary to avoid depriving the water of its oxygen, since when a gas bubbles through a liquid it removes other gases. The water had to be vigorously shaken with air after the carbon dioxide had been added. A good deal of manipulation is required when working with higher concentrations of carbon dioxide to secure the proper amount of oxygen and carbon dioxide simultaneously in the water. In all of the experiments reported the oxygen content of the water was ample. It may be noted in passing that a combination of low oxygen with high carbon dioxide has a much greater depressing effect than either of these conditions separately. Under ordinary conditions the rate of oxygen consumption is normal in water containing 2 cc. per liter but oxygen consumption is almost abolished in such water if it be saturated with carbon dioxide. When plenty of oxygen is present, the depression produced by saturated carbon dioxide is only about 50 per cent.

Most of the experiments with carbon dioxide are reported in Table V. In the more dilute solutions—pH 7.5 to 7.0—carbon dioxide acidity produces less depression than do other mineral

[1] Animals injured.

acids. In fact, in these concentrations, there is a tendency towards stimulation of the respiratory rate. Out of nine experiments, stimulations of 2 to 12 per cent. were obtained in five cases. The amount of stimulation, however, lies within the experimental variation. The truth probably is that carbon dioxide acidity does not affect the rate of oxygen consumption until the acidity reaches a greater degree than pH 7.0. At greater acidities than pH 7.0, the rate of oxygen consumption is depressed and to a degree similar to that produced by other mineral acids. It was not possible to obtain an acidity with carbon dioxide greater than pH 5.2 with the well water, owing probably to the buffers present in the water.

7. *Experiments with Acetic and Butyric Acids.*—These two acids were selected as examples of the paraffin acids, supposed to penetrate organisms readily. It was anticipated that the depressing effect of these acids would be greater than that of mineral acids. Such, however, was not the case, but the contrary result appeared. Acetic acid, in acidities between 7.8 and 7.0 tended to stimulate the rate of oxygen consumption. Of six experiments performed at pH 7.5, the rate of oxygen consumption was unaffected in two cases, stimulated in two, and depressed in the remaining two. Of six experiments at pH 7.0, all but one were stimulating. It seems probable that low concentrations of acetic acid accelerate the rate of oxygen consumption. At concentrations greater than pH 7.0, all concentrations depressed the rate of oxygen consumption during the first hour's exposure. During later exposures, a tendency toward acceleration was again manifest. These facts are brought out in Table V. and VI. Butyric acid was the least effective in inducing depression of all of the acids used. This result was very surprising and still remains inexplicable. Butyric acid has only a slight depressing action at all concentrations between pH 7.5 and 5.5. At pH 5.0 a depression of 30 to 40 per cent. appears but this concentration of butyric acid is lethal and the worms begin to die within an hour. Acetic acid at pH 5.0 is also injurious. The data on butyric acid are summarized in Table VI., which gives the initial depression induced. Later exposures did not alter the result.

8. *Experiments with Tartaric and Citric Acids.*—The former was

selected as an example of a dibasic, the latter of a tribasic acid. The results were not of particular interest and are summarized in the general table, Table VI. The action of tartaric acid was very similar to that of the mineral acids. Citric acid was less effective than the inorganic acids at concentrations lower than pH 7.0.

9. *Consideration of Other Factors.*—(*a*) *Size.* It was found long ago by Child that young (small) individuals are more susceptible to lethal concentrations of various substances than are old (large) individuals of a given species. MacArthur ('20) studied the relation between age (size) and susceptibility to acids in *Planaria dorotocephala.* He found that in concentrations of hydrochloric and other acids which are quickly lethal, between pH 2.0 and 4.5, the young (small) individuals die sooner than the old (large) ones. At slightly lower concentrations, pH 4.7 to 4.9 for HCl, the relation is reversed, the larger individuals succumbing first. In still weaker solutions, planarians live indefinitely. These results have been repeatedly confirmed in this laboratory in class work, although of course the precise concentrations required to yield the results mentioned are subject to variation, owing chiefly to differences in the physiological condition of the worms themselves.

Since according to these results, small worms are more susceptible to acids than large worms at concentrations greater than pH 4.5, it seems probable that the percentage of depression induced by acids should bear some relation to size. This was tested in a number of experiments, in which the depressing action of HCl on large and on small worms was compared. Only recently collected material was used as the metabolic differences between worms of different sizes are greater in such material. The heads of all worms were removed before the test, in order to eliminate movement, as small worms are more active than large ones. Decapitated worms are very inactive. The small worms used were under 10 mm. in length, the large ones over 20 mm. Twelve experiments were performed at concentrations of HCl ranging from pH 3.6 to 4.6. In ten of these the percentage of depression was greater in the small than in the large worms. The difference ranged from 3 to 20 per cent. In two cases the

result was reversed, the larger worms exhibiting about 10 per cent. more depression than the small ones. In general, then, the experiments indicate that in concentrations which will kill within a few hours and in which small worms die slightly faster than large worms, the small worms are more greatly depressed by the acid. It should be added that the animals suffered no injury during the period of exposure and recovered completely.

(*b*) *Previous History with Respect to Acid.*—From my experiments I have gained the distinct impression that the amount of depression induced by exposure to acidified water is to some extent dependent on the previous history with respect to acid. If a given lot of worms, which has never been exposed to acidified water, is tested at rather high acidity, say pH 5.0, it is commonly found that the amount of depression is much less than would ordinarily be expected at that acidity. If on the other hand, such worms are gradually accustomed to acidified water, by exposing them first to pH 7.5 and gradually increasing the acidity, then a much greater percentage of depression is obtained at high acidities. One of the most striking cases of this kind in my investigation occurred in working with sulphuric acid. Three lots of worms, never before exposed to acidified water, were tested in water acidified to pH 5.0 with sulphuric acid. The percentages of depression obtained were 20, 30, and 15 respectively. The same lots of worms were then exposed on successive days to various concentrations of sulphuric and hydrochloric acid, beginning at 7.5 and gradually working down to pH 5.0 again. When sulphuric acid pH 5.0 was then again tried, on the same worms, the depressions obtained were respectively 39, 56, and 45 per cent. The same type of result was frequently met with. It appears that when worms are suddenly exposed to rather strongly acidified water, they are able to produce or manufacture some substance which protects them from the acid. But after repeated exposures to acidified water, the substance is either exhausted or the worms have become so accustomed to residing in acidified water that they no longer respond to it by producing the substance. Sudden exposure to acid may act as a stimulus to production of basic substances; repeated exposure fails to stimulate.

It seems probable that in this behavior of the worms is to be found the explanation of the numerous puzzling variations in the degree of depression obtained.

(*c*) *Acclimation.*—It was my intention at the beginning of this work to determine whether the animals could recover from the depression induced by acid if allowed to remain for some time in the acidified solution. I found, however, that the experiment is impractical, because of the carbonate content of the water used. When the water is acidified it soon becomes alkaline and unless acidified to a point which would be fatal to the worms, returns to an alkaline condition within 24 hours. Consequently if worms are placed in the acidified water at a certain pH, the water does not remain at this pH, but the pH rises (the acidity falls). If then the worms are tested at the new pH, the depression is naturally less than it was at the beginning of the exposure. If tested at the original pH, the depression is greater than at the pH attained by the standing solution. In brief, it is impossible to determine the effect of long continued exposure to a given pH, unless the water is freshly acidified and changed every hour, for two or three days. As this is physically impossible, for me at least, the experiment had to be abandoned. I have, however, a number of experiments, especially with HCl, in which the oxygen consumption was tested during the first, third, and fifth hours of continuous exposure to a given concentration. Some of these data appear in Table IV. In general it was found that there is very slight if any recovery during successive hours of exposure. After this had been repeatedly determined, the tests of the later exposures were abandoned.

The reader will no doubt at once inquire why carbonate-free water was not employed in a study of acclimation. The reason for this as will appear later is that acidified carbonate-free water does not affect the oxygen consumption of Planaria.

10. *General Results and Discussion.*—We may now state and discuss the results obtained with acidified unaltered water.

(*a*) Acidification of the water generally causes a depression of the rate of oxygen consumption. This was true of the three mineral acids, hydrochloric, sulphuric, and nitric, at all concentrations employed. Even a change from the normal pH 8.0 of

the water to 7.8 by addition of such acids induces a measurable lowering of the rate of oxygen consumption. In the case of some acids, notably acetic, less so with carbonic, there was some tendency to an acceleration or stimulation of the oxygen consumption at acidities between 7.8 and 7.0. Yet this effect was so slight, and the variability of the results so great that little emphasis can be placed upon this finding, unless a very large number of experiments were carried out. All of the acids used caused depression at all acidities greater than pH 7.0, but the action of butyric was very slight, almost nil.

(*b*) The percentage of depression increases with increasing acidity within certain limits.

(*c*) The lower concentrations are, however, relatively more effective than the higher concentrations. This is generally the case wherever chemicals are applied to living organisms. The general form of the curve obtained is seen in Fig. 1. This type of curve is so commonly obtained in physiological experimentation that it must possess some deep significance. I am unable however to suggest any explanation of this type of curve.

(*d*) The inorganic acids, except carbonic, are in general more effective than the organic, particularly in the lower concentrations, pH 7.5 to 6.5. This finding was the contrary of my expectations and contrary to the results of Gray ('24). It is generally believed that organic acids, such as acetic and butyric, penetrate protoplasm readily, while mineral acids are unable to do so. Obviously one would then expect the organic acids to act more powerfully on respiratory metabolism than the inorganic acids. As this was not found to be the case, it must be concluded that the penetrability of the acid has no bearing on the result. The lack of action of butyric acid remains inexplicable.

(*e*) The hydrogen ion concentration cannot be the chief cause of the depression induced, because different acids do not produce the same percentage of depression at the same pH. The result does not substantiate the contentions of Loeb ('22) that the action of acids on colloids depends only on valence and hydrogen ion concentration. According to Loeb's ideas, all of the monovalent acids, such as hydrochloric, nitric, acetic, and butyric

should have produced the same amount of depression at the same hydrogen ion concentration. Reference to Table VI. shows that this is not at all the case, butyric acid furnishing a notable exception. According to Loeb also, di- and tri-basic acids should be less effective than monobasic. This again is not upheld by my results. Sulphuric acid is even more effective than hydrochloric, although being dibasic it should be only half as effective. Tartaric, another dibasic acid, has about the same efficiency as the monobasic mineral acids. Of course, it is not certain that Loeb intended his ideas to apply to living organisms. Further the hydrogen ion concentrations with which Loeb worked are mostly instantly or rapidly fatal to living organisms. It remains to be proved whether the statements of Loeb will hold at physiological concentrations of acid. I have been informed that Michaelis has publicly stated that they do not hold and that the Hofmeister series remains unshaken. From the fact that in my experiments different acids produce different degrees of depression at the same hydrogen ion concentration it appears necessary to conclude that the hydrogen ion concentration is not the principal factor in the result.

(*f*) When a certain degree of acidification has been attained, further acidification does not increase the percentage of depression. The percentage of depression obtained at pH 5.0 is about the maximum that can be produced without actual injury to the animals. It appears that a depression of 50 per cent. is the most that can be obtained with any acid on the average. Of course individual experiments may yield a depression slightly greater than this. A depression of 58 per cent. is the greatest recorded in the dozens of experiments performed with the various acids and this figure was obtained on young worms. The statements in this paragraph apply only when an ample supply of oxygen is present in the water.

(*g*) The decrease in the rate of oxygen consumption reaches its maximum value for any particular concentration during the first hour of exposure to the acidified water. Prolonged exposure does not increase the depressing effect. On the contrary there is generally some slight rise in the oxygen consumption during several hours exposure. This is too small however to be considered of significance.

(*h*) The depressing action of acids is completely and promptly reversible, wherever an actual injury to the tissues of the animal has been avoided. Recovery occurs almost immediately, commonly within the first hour after return to normal water. Several experiments were devised to test the possibility that the decreased oxygen consumption while in the acid might be compensated for by an increase over the normal during the period immediately upon return to normal water. In these experiments the oxygen consumption was first tested in normal water, then in acidified water, of a concentration to give at least 30 per cent. depression, then immediately in normal water again. In most of these cases the respiration had risen to the normal value during the first hour after return to normal water. In a few cases, the oxygen consumption was below normal. In no case was any rise over the normal figure observed.

(*i*) The experiments justify the use of acids as agents for experimentally producing a state of depression. They also substantiate the generally held view that the effects produced by acids on such processes as cleavage, development, and regeneration are assignable to a reduction in the rate of respiratory metabolism.

## Experiments with *Planaria* in Carbonate-free Fresh Water.

The experiments had reached the point outlined above by the summer of 1924 and I intended to bring the investigation to a close. A number of matters puzzled me greatly but I was unable to devise any means of throwing further light on them. At about this time, however, my attention was drawn to the experiments of Clowes and his associates ('23, '24) in which it was shown that the carbon dioxide set free by acidification of sea-water markedly influences the result and is in some cases the real agent involved. At first I was not inclined to believe that carbon dioxide was responsible for the results which I had obtained with acids. It seemed to me that if carbon dioxide were chiefly or wholly responsible for the observed effects, carbon dioxide acidity should be more effective than acidities produced by other acids and the action of various acids should be similar

at the same hydrogen ion concentrations. As these conditions did not obtain in my experiments, I considered it unlikely that the findings of Clowes applied to them. It seemed necessary to me, however, that the matter should be tested by experiment. A new series of experiments was therefore begun in the fall of 1924 using carbonate-free water. The results obtained convinced me that the depressing action of acidified water is largely due to the carbon dioxide liberated in it.

In connection with these experiments a large number of trials in ordinary carbonate-containing water were carried out as controls. These constituted repetitions of the work of the previous year. It was very puzzling to me to find that all acids were less effective than had been the case in the preceding year. This was particularly noticeable at the lower concentrations, pH 7.5 to 7.0. The mineral acids produced about the same depressing effect as previously at the higher concentrations, 6.5 to 5.0. The organic acids (acetic and butyric were the only ones tried) were also markedly less effective than had previously been the case, at all concentrations tried. I am unable to explain this state of affairs except on the assumption that the carbonate content of the water had decreased in the meantime. The fact that in many cases the controls gave a smaller percentage of depression than previously made it difficult to arrive at conclusive results.

1. *Preparation of Carbonate-free Water.*—Carbonate-free water was prepared according to the method of Smith and Clowes ('24*b*). Two cc. of concentrated hydrochloric acid were added to eight liters of the well water in a large bottle. Air from the compressed air system was passed through the water in the bottle for 24 hours or more. This treatment was found to remove the carbonate from the water completely. Several times liter samples of this water were evaporated to dryness and the residue tested for carbonate with entirely negative results. In some cases borax was added to the carbonate-free water to serve as buffer but as the results were not affected by this procedure, it was generally omitted. The oxygen consumption of the worms in this carbonate-free water, with or without borax, was repeatedly compared with that in the normal unaltered well water. No difference was found.

2. *Action of Mineral Acids Added to Carbonate-free Water on the Rate of Oxygen Consumption.*—The same amount of acid was added to the water as would be required to produce the desired pH in unaltered water. Alkali was then added until the pH in question had been attained. Rather extensive experiments were performed with hydrochloric acid, less extensive with sulphuric and nitric acids. The results in all three acids were the same and were very striking. Acidification of carbonate-free water with these acids has *absolutely no effect* on the rate of oxygen consumption of Planaria at all concentrations between pH 7.5 and 5.0. At pH 4.0, a slight depressing effect was noted, about 15 per cent. This result is graphed in Fig. 1.

It is thus proved that practically the entire depressing effect on the oxygen consumption of *Planaria* produced by the addition of mineral acids to natural water arises from the carbon dioxide set free in such water by decomposition of its contained carbonates by the acid added. The depressing effect is thus a carbon dioxide depression. It is probable that the carbon dioxide penetrates the animal in the gaseous state and acts within the animal as such or by inducing an internal acidity (cf. Jacobs, '20).

3. *Action of Organic Acids in Carbonate-free Water.*—Only acetic and butyric were tried. I was particularly interested in testing these two acids as they are believed to penetrate organisms readily. The result should serve to indicate whether the efficiency of carbon dioxide is merely a question of penetrability. Unfortunately decisive results could not be secured, owing to the fact, already mentioned, that very little depressing effect was obtained in the controls. Butyric acid, previously found to be the least effective of all of the acids tried, was in this series of experiments quite ineffective in normal water. Even at pH 5.0, an injurious concentration, no depression of the oxygen consumption in normal water appeared. Consequently the action of butyric acid in carbonate-free water could not be determined. With acetic acid, some results were secured. Acetic acid was about half as effective in inducing depression as in the experiments of the preceding year. At concentrations weaker than pH 7.0 there were again indications of acceleration of the rate of oxygen consumption in the controls. At acidities of pH 6.5

or greater, depression was produced in normal water, but to a less extent than previously. In the same worms, in carbonate-free water acidified with acetic acid, the depression induced was noticeably less than in the controls. It therefore follows that the depressing action of acetic acid is also in large part due to the carbon dioxide which is sets free. It appears that the penetrating powers of carbon dioxide do not entirely explain its difference from other acids.

4. *Action of Carbon Dioxide Added to Carbonate-free Water.*—These experiments were designed as a sort of crucial test of the proposition that carbon dioxide is the cause of the depression induced by acidification of natural waters. The proposition was upheld in the most striking manner. The addition of carbon dioxide gas to the same pH causes the same degree of depression, whether added to normal, or to carbonate-free water. It thus appears to be reasonably certain that the depression of the rate of oxygen consumption in acidified water is caused chiefly by carbon dioxide.

The question then arises: Is the amount of carbon dioxide liberated in carbonate-containing water by the addition thereto of acids, different with different acids at the same hydrogen ion concentration? It became necessary to determine the actual amount of free carbon dioxide present when the well water was acidified with various acids.

5. *Determinations of the Amount of Carbon Dioxide Liberated on Acidification of the Unaltered Water.*—The normal well water, pH 8.0, was acidified to the desired pH with various acids. A sample of 100 cc. was then immediately drawn and the free carbon dioxide in it determined by titration with $N/50$ barium hydroxide, properly protected from the carbon dioxide of the air, using phenolphthalein as indicator.

Hydrochloric, sulphuric, acetic, and butyric acids were investigated at ranges of pH 7.5 to 4.0. It was found that the amounts of carbon dioxide liberated from the carbonates of the water by different acids are similar, but not identical, at the same pH. Such differences probably account for some of the different percentages of depression obtained with different acids at the same pH. But butyric acid was also found to liberate nearly as

much carbon dioxide at the same pH as does hydrochloric acid. The lack of depressing action of butyric acid therefore remains at present inexplicable.

Nearly all of the carbonate of the water is liberated as carbon dioxide at a pH of 5.0 and all of it between pH 5.0 and 4.0. The amount of free carbon dioxide present at these acidities is about 30 cc. per liter. At first the fact that all of the available carbonate in the water is decomposed at pH 5.0 to 4.0 appeared to explain the result that the maximum percentage of depression is obtained at such acidity and further acidification does not increase the depression. But the additional fact discussed in the next paragraph that increasing the amount of carbon dioxide by adding the gas is not effective in intensifying the depression invalidates the suggested explanation.

Analyses were also made of the amount of carbon dioxide gas required to produce a certain pH. It was found that more of the gas is present in the water at a given pH than is set free in the water by adding other acids to the same pH. This result would be expected, since it is highly probable that when the water is acidified, considerable time must elapse before all of the acid reacts with the carbonate of the water. Consequently immediately after acidification part of the free hydrogen ions present are derived from the acid added, and not from the reaction of carbon dioxide with water. The difference in carbon dioxide content between water acidified with carbon dioxide gas and water acidified by other acids is small at pH 7.0 or even 6.5 but at pH 6.0 there is three times and at pH 5.0 nearly ten times as much carbon dioxide gas in the water as is liberated by mineral acids at those same hydrogen ion concentrations. These results are shown in Fig. 2.

We are thus faced with the question—why is carbon dioxide not more effective as a depressing agent than any other acid, particularly at the higher acidities? There was to be sure evidence that carbon dioxide at pH 5.0 for instance has a greater depressing action than any other acid tried. Yet the difference between it and other acids is not as great as might be expected. It becomes necessary to assume, in view of the facts at hand, that the amount of depression of the respiratory rate which can

be induced in Planaria by carbon dioxide is limited and does not exceed 50 per cent. as long as the oxygen supply remains ample. If the oxygen supply is reduced, although not to a point where the respiration rate would be affected under ordinary conditions,

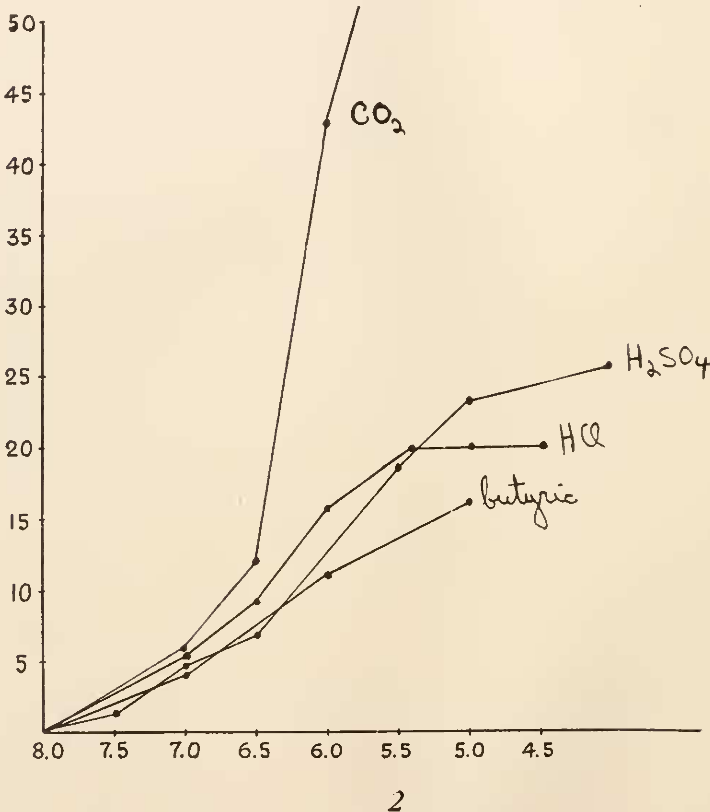


FIG. 2. Graph of the amount of carbon dioxide liberated in the well water by various acids. Amount of carbon dioxide in cc. per liter on the ordinate, pH on the abscissa. The amount of carbon dioxide gas required to produce a given pH is also shown.

the oxygen consumption can be nearly abolished if the water is saturated or nearly so with carbon dioxide. A carbon dioxide content of 30 cc. per liter appears to yield the maximum depressing effect, about 50 per cent., that can be obtained with carbon dioxide, when the oxygen supply is adequate.

6. *Conclusions Concerning the Experiments with Carbonate-free Water.*—(*a*) The depressing effect of acids on the oxygen consumption of Planaria is almost wholly abolished when carbonate-free water is used, except when the acidity is produced by carbon dioxide gas.

(*b*) An acidity due to carbon dioxide gas is equally depressing in ordinary and in carbonate-free water.

(*c*) From the statements in (*a*) and (*b*) it is concluded that the depressing action of acids on oxygen consumption is almost wholly due to the carbon dioxide liberated on acidification of natural waters.

(*d*) Different acids at the same hydrogen ion concentration do not immediately liberate the same amounts of carbon dioxide from the natural carbonate-containing water. This accounts in large part for the fact that the depressing action of different acids is not the same in degree at the same pH.

(*e*) All of the carbonate of the water used is decomposed at a pH of 4.0 to 5.0, giving a carbon dioxide content of about 3 per cent.

(*f*) The lack of action of butyric acid is not explained, for this acid also liberates carbon dioxide from the carbonates of the water. Possibly butyric acid in some way prevents the penetration of carbon dioxide into the animals.

(*g*) The amount of carbon dioxide gas required to produce a given pH is considerably greater at acidities of more than pH 6.5 than is the amount of carbon dioxide liberated in the water by other acids at the same pH. One would therefore expect that $CO_2$ would be much more effective as a depressing agent at acidities from 6.5 to 5.0 than any other acids. This was not noticeably the case. The difficulty could be explained by assuming that the depressing action of $CO_2$ reaches a maximum at a concentration of 3 per cent. and is not altered by further increase in the concentration of the gas.

(*h*) Since the depressing effect of acids is due largely to carbon dioxide and since this gas is an end product of metabolism, it becomes readily understood that organisms or parts must be affected by acids in relation to their respiratory metabolism. The higher the rate of the latter, the greater would be the depression induced by acids.

## Relative Toxicity of Different Acids.

An attempt was made to discover the hydrogen ion concentration at which each of the acids used would cause the death of *Planaria* within a relatively short period of time, say, two to three hours. A sort of trial and error procedure is necessary to determine this matter. The results are not very exact but the relative toxicities of the different acids were plain enough after a few trials. Butyric acid is by far the most toxic, as found also by other investigators, and kills the animals within two or three hours at pH 5.0. Acetic acid ranks next in toxicity, killing in the time specified at pH 4.4. Tartaric and citric acids come next, killing within two or three hours at 3.6 and 3.4 respectively. The three mineral acids are nearly equally toxic, sulphuric being slightly more effective, killing in two or three hours at pH 3.2 to 3.4, while hydrochloric and nitric acids must be used at pH 3.0 to 3.2 to obtain the same result. The order of toxicity may then be expressed as follows: butyric > acetic > tartaric > citric > sulphuric > nitric = hydrochloric.

Carbon dioxide at saturation (about pH 5.2) was not found to kill the animals as long as the oxygen supply was adequate.

The death of the animals took place with equal rapidity and at the same hydrogen ion concentrations in both normal and carbonate-free water. This proves that the death is not due to the carbon dioxide set free by acids in normal water but is a direct acid effect. This is further evidenced by differences in the appearance and in the manner of disintegration of the dead animals. Death in acids appears to be generally due to coagulation. This was also noted by Mrs. Lewis, '23.

In view of the fact that the death of the animals results from a direct coagulating action of the acids while the depression of the respiratory rate by acids is caused almost wholly by carbon dioxide, no relation would be expected between the toxicity of different acids and their depressing action. This is the case. Butyric acid, the most toxic, is in non-lethal doses the poorest depressant. The experiments do not of course serve to show the amount of depression at or near the death point, except in the cases of acetic and butyric acids, pH 5.0, Table VI. Since different acids are equally lethal at different hydrogen ion

concentrations, it follows that the free hydrogen ions cannot be the chief factor in toxicity. The penetrating powers of the acid are probably of great importance and the nature of the anion or molecule may also be involved. Butyric and acetic acids which probably penetrate organisms the most readily of all the acids employed are also the most toxic. In a study of the toxicity of various acids for ciliate Protozoa, Collett ('19) reached the conclusion that hydrogen ion concentration is not the most important factor.

## Summary.

1. The effect of acidification of the medium on the rate of oxygen consumption of aquatic organisms was studied.

2. The acids used were: hydrochloric, nitric, sulphuric, carbonic, butyric, acetic, citric, and tartaric. They were added to water to produce acidities ranging from pH 7.5 to 5.0, at intervals of 0.5 pH.

3. *Planaria dorotocephala* was the chief animal used as material. Some tests with hydrochloric acid were also made using starfish and nudibranchs.

4. The acidification of natural waters, either salt or fresh, (pH 8.0), by any of the acids used except butyric causes a decrease in the rate of oxygen consumption at all acidities greater than pH 7.0. The majority of acids also cause depression between 7.8 and 7.0 but in the case of acetic acid and more doubtfully carbonic, there was some tendency towards a slight acceleration of the rate of oxygen consumption at these lower concentrations.

5. The decrease in the rate of oxygen consumption due to acids is completely and promptly reversible, as long as the animals are not actually injured.

6. The acidification of fresh water from which all carbonates have been previously removed has no or only a slight effect upon the rate of oxygen consumption of *Planaria*, except when the acidity is produced by carbon dioxide.

7. The depressing action of carbon dioxide is the same whether the gas is added to ordinary or to carbonate-free water.

8. From 6 and 7 it follows that the depressing action of acids in natural waters is due chiefly or wholly to the carbon dioxide which they liberate from the carbonates of such waters.

9. The depressing action of acidified natural waters on the rate of oxygen consumption of *Planaria* is not the same with different acids at the same hydrogen ion concentration. This appears to be due largely to the fact that the amount of carbon dioxide immediately liberated from the carbonates of the water differs with different acids at the same pH.

10. The depressing action of acids in natural waters is greater the greater the acidity up to an acidity of about pH 5.0. This is due to the fact that the more acid added, the greater is the quantity of carbon dioxide liberated.

11. Lower concentrations are, however, relatively more effective than higher ones.

12. The maximum amount of depression of oxygen consumption that can be induced by acids is about 50 per cent. This occurs at pH 4.0 to 5.0 and further acidification of the water does not increase the percentage of depression. At the acidity at which the maximum depression appears, the carbon dioxide content is about 3 per cent.

13. All of the carbonates of the fresh water employed are decomposed by acids at a pH of 4.0 to 5.0, producing a carbon dioxide concentration of 3 per cent. This might explain the facts given in 12 were it not that concentrations of carbon dioxide gas much higher than 3 per cent. do not increase the percentage of depression beyond 50 per cent., as long as the oxygen supply is ample. Concentrations of carbon dioxide gas up to 25 per cent. were tested.

14. From the facts cited in 13 it appears necessary to assume that the depression of the rate of oxygen consumption which can be induced by carbon dioxide does not exceed 50 per cent. as long as the oxygen supply is adequate.

15. A combination of high carbon dioxide content and low oxygen content practically abolishes the oxygen consumption of *Planaria*, even though the oxygen content used (2 cc. per liter) would be ample for normal respiration in the absence of carbon dioxide.

16. No explanation has been discovered for the differences between the action of carbon dioxide and other acids at low concentrations (pH 7.5 and 7.0).

17. Butyric acid has almost no action on the oxygen consumption of *Planaria*. No explanation has been discovered for this fact.

18. The different acids employed are lethal for *Planaria* in a given arbitrarily selected time (2 to 3 hours) at different hydrogen ion concentrations. The order of toxicity and the hydrogen ion concentrations at which the acids are equally lethal are: butyric (5.0), acetic (4.4), tartaric (3.6), citric (3.4), sulphuric (3.2) and nitric and hydrochloric (3.0).

19. The facts given in 18 prove that the hydrogen ion is not the cause of death but either the anion or the molecule of the acid is involved. Penetrability is probably also a factor. Death in acids appears to be due to coagulation.

20. The order of toxicity of the acids and the pH at which they are equally lethal are the same in ordinary and in carbonate-free water, showing that death is not due to carbon dioxide liberated.

21. Acidification of natural waters constitutes a method for depressing the rate of oxygen consumption of aquatic animals for experimental purposes.

22. The experiments herein presented cast doubt on the supposed importance of hydrogen ion concentration *per se* in biological processes.

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*Vol. XLIX* *November, 1925* *No. 5*


# BIOLOGICAL BULLETIN


## THE RELATION OF *HERPETOMONAS ELMASSIANI* (MIGONE) TO ITS PLANT AND INSECT HOSTS.

FRANCIS O. HOLMES.[1]

*Herpetomonas elmassiani* (Migone) is at present the only species of latex-inhabiting herpetomonad known in the United States. Its plant host is the common milkweed, *Asclepias syriaca* L., in the latex of which it has been found in great numbers in Maryland and New Jersey (Holmes, 1924, 1925*a*). Its suspected insect host in these locations is a red and black hemipterous insect, *Oncopeltus fasciatus* (Dall.). The same species of plant flagellate appears to inhabit other milkweeds in countries along the Atlantic coast of Central and South America. It has been reported from Haiti, Honduras and Paraguay, and doubtless exists at intermediate points between these countries and the locations in the United States. In southern locations other species of *Oncopeltus* frequenting the infected plants have seemed to act as insect hosts.

In view of the lack of apparent pathogenicity of the herpetomonads in Maryland milkweeds (Holmes, 1925*b*), it seems desirable to report upon the relation of the flagellate to the tissues of its hosts. The well known species, *Herpetomonas davidi* (Lafont), which inhabits the latex cells of Euphorbias in Europe and elsewhere, is pathogenic to its host (França, 1914, Nieschulz, 1922), in which it causes modifications of the latex cells and neighboring portions of the plant sufficient to stunt or kill whole branches or even whole plants. The reasons for the lack of harm resulting in the milkweed host from the presence of *Herpetomonas elmassiani* (Migone) may be the freedom from infection of some of the latex systems even of heavily infected

[1] Joint contribution from the laboratories of the Boyce Thompson Institute for Plant Research and of the Department of Medical Zoölogy, School of Hygiene and Public Health, Johns Hopkins University.

plants, the entire freedom from penetration of tissues aside from the latex systems, and the sufficient food supply presented to the flagellates by the milky vacuole fluid itself in the latex cells.

The fact is often overlooked that plant flagellates of the type of *Herpetomonas davidi* (Lafont) and *Herpetomonas elmassiani* (Migone) have been found *only* in such plants as have abundant latex. Those engaged in research in this field are of course acquainted with this restriction of the range of the organisms, and take it into account in most of their work, but do not always seem to have it very definitely in mind. Others whose interest in plant flagellates arises from some other, less immediate, source are often entirely unmindful of the situation until it is brought to their attention.

To the present time no acceptable species of flagellates of the genera *Herpetomonas*, *Leishmania*, *Crithidia* or *Trypanosoma* have been found in plants other than those provided with a milky juice or latex.

Perhaps the situation would be better understood if it were commonly known that latex does not occur extracellularly in plants, but intracellularly. Thus the flagellates which are transferred by their insect hosts to the latex cells of plants are not to be found thereafter at random in the plant tissues, but are strictly intracellular (not intracytoplasmic) parasites.

A description of the cells containing the latex will make clear the relation of any latex-inhabiting organisms to the host plants.

The latex ducts of the plants with which protozoölogical studies have been most concerned are those known as simple ducts, because they do not fuse with each other in the course of their wanderings. Among the Asclepiadaceæ, Euphorbiaceæ, Apocynaceæ and Urticaceæ the cells destined to become the latex ducts of the mature plants are already distinguishable in the embryo. Their nuclei divide again and again, and the cells elongate tremendously and branch repeatedly, but no cross walls are formed, nor do the ducts fuse with one another, so that eventually the few original cells penetrate every part of the plant, and still remain distinct and separate from each other. The thin cellulose walls are lined with a layer of cytoplasm containing numerous nuclei. In the vacuole is collected the milky, usually white latex.

It is well to bear in mind that there is another type of latex system among plants, in which the original cells fuse together by the destruction of their partitions and cross-walls. The result

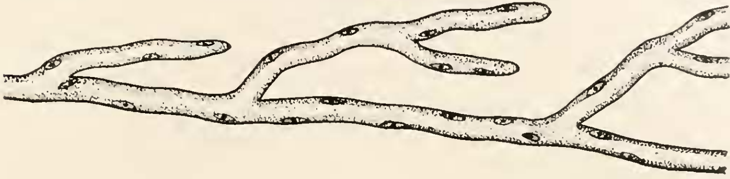

FIG. 1. Diagrammatic representation of a portion of a simple latex duct such as is found in the species of the genus *Asclepias*. The latex is secreted by the wall of cytoplasm into the extensive central vacuole. Such a duct will not fuse with others in the course of its growth.

of the process is the formation of interlacing vessels, in which the latex is contained. This type of latex system is found in such plants as lettuce.

Diagrams representing these two types of latex ducts are shown in Figs. 1 and 2.

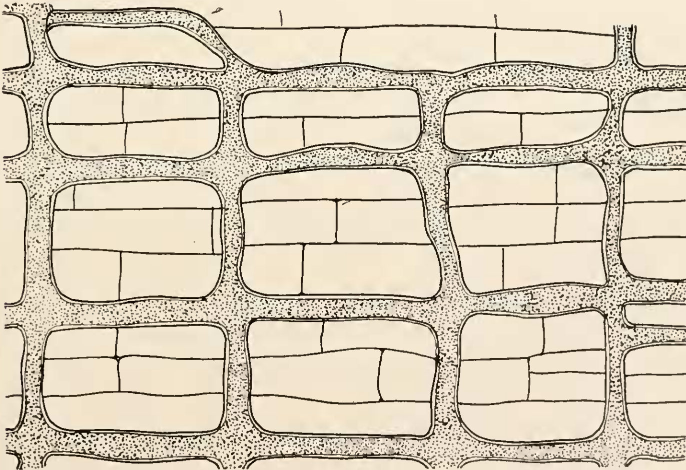

FIG. 2. Diagrammatic representation of a system of latex vessels such as is found in lettuce. The original cells fuse together to form a network, instead of remaining as independent latex ducts. The type of latex cell involved in protozoölogical studies has been that shown in Fig. 1.

The flagellates known to inhabit the latex systems of plants are probably all insect parasites. They enter the cells of the

plant hosts only when the insects introduce their beaks directly into the latex to feed. In the cells they do not enter the cytoplasm, so far as can be seen, but inhabit the milky juice which fills the long cell vacuole. There they grow and multiply rapidly, as is indicated by the many dividing specimens present in latex smears from infected plants stained with Wright's stain. The flagellates are shut off from other cells of the plant and even from other latex cells of which there may be several. Unless a single plant were infected several times by insects it is unlikely that all the latex ducts would become infected, even though one or more might.

### Localized Infections.

If one latex system of a plant is infected, a macroscopically localized infection may result unless that system happens to penetrate every leaf and flowerlet of the plant.

Early in the season of 1924 a search was made for such cases, for the previous year all the infections had appeared systemic.

It seemed likely that insects might repeatedly bite plants during the late summer, but that during June and early July any infections which might occur from insect carriage would be the result of a minimum number of infective bites.

The first example of the way in which this worked out in the field was met when a plant of a group surveyed thoroughly every few days gave a negative record after showing flagellates on several occasions. The later examinations of this plant showed that flagellates were present, but were not always to be found in the single drops of latex preserved as records. For this plant, then, a new system of sampling was instituted. Samples were taken from ten leaves instead of from one. It was found that some leaves were positive and others negative, just as one might reasonably expect if only a few of the latex cells were parasitized.

The study of sections of petiole and leaf tissue gathered at this time showed the even more interesting fact that in plants never suspected of having localized infections only a few of the latex cells were inhabited by organisms. It was easier to find negative cells than positive in specimens from apparently heavily parasitized plants. Smears from these plants showed very large

numbers of organisms, yet when a droplet was taken for examination as much latex must have been contributed by uninfected cells as by infected. In the cells containing the organisms the concentration was so great that a moderate dilution was entirely ineffective in changing the appearance of the drop as stained for examination.

Since the plants above mentioned seemed when in the field to have systemic infections in that the slightest wound in any portion of the leaves or stems gave infected latex, and yet the microscopic examination of sections showed but a few of the cells infected, it is evident that the whole plants were penetrated by a relatively few single cells, and that if each individual latex cell fell short of extending completely throughout its plant, it still must have achieved very nearly this remarkable feat. This type of cell attains a notable total size and contains tremendous numbers of nuclei. Its latex-containing vacuole also is probably nearly if not quite the largest of cell vacuoles, making up in length far more than it lacks in breadth.

## Confinement to Latex Cells.

Careful study failed to show any flagellates in the plant tissues outside the vacuoles of the latex cells. In some sections there were indeed abnormal and deceptive appearances caused by the flow of latex by capillary action along the conducting tissues. When pieces were cut to be fixed, latex always flowed out over the wounded surfaces. Since the turgidity of the plant was in part relieved by this loss, other specialized cells such as the spiral vessels were invaded by currents of latex along with which the flagellates themselves were carried. The abnormality of this process was made evident by examining the invaded ducts throughout their entire length. The flagellates were seen to be present only near the cut surfaces and to become less and less crowded as the distance from the exterior increased. The tissues in the interior presented a truer picture of conditions in the living plant.

Since the flagellates were normally confined to the latex cells, and indeed to the *latex* filling the long vacuoles, it became evident that certain observations made in the field were of more signifi-

cance in their relation to the biology of the flagellate than had at first been realized.

It had been noticed that infections did not spread from plant to plant in groups connected by a common axis. Such plants arose from separate buds, the latex systems of which were independent of each other. In the axis itself no ducts occur, so that there is no chance for wandering through such a connection.

Late in the autumn of 1924 two plants which had been under observation for months were dug up to determine their exact relation to each other. One of these had been consistently negative for flagellates all summer. The other had been as consistently heavily infected in every part above the ground, with the exception of the seeds which are always free from invasion. The two plants were separated from each other by no more than six inches of axis, from which common source they had both sprung as buds. The absence of latex ducts in the axis and the confinement of the flagellates to the latex of the infected plant made it impossible that the nearby negative plant should be invaded except from some outside source of the organisms.

The practical confinement of the insect, *Oncopeltus fasciatus* (Dall.), suspected of being the insect host of the flagellate, to the blossoms and pods of the milkweed plant in feeding is also made significant by histological studies of the latex system, which in these two parts becomes much more prominent than it is in the stems or leaves. The soft tissues under the outer green coverings of the pods contain numerous branches of the latex system, and in the area between the pedicels and the bases of the petals of the flowers the latex ducts are exceedingly close together and voluminous. This offers a favorable feeding location to the insect, and, by reason of the crowded flagellates here in infected plants and the softness of the tissues, opportunity is offered for the infection of insects from the plant and for the infection of previously uninfected plants by the insects.

## The Flagellates of *Oncopeltus fasciatus* (Dall.).

It is not yet known with certainty whether *Oncopeltus fasciatus* (Dall.) is the insect which carries the milkweed flagellate from

one plant to another. But since there are several reasons for suspecting it to be the carrier, a study of its flagellates was undertaken. All along the coast of America, from Paraguay in the south to New Jersey in the north, wherever the flagellate has been found in the latex of the milkweeds some species of the genus *Oncopeltus* has been found feeding on the infected plants. These insects have always been more characteristic of the particular plants harboring the flagellates than any other type of insect. Just north of New Jersey, where no flagellates are known in the plants, another insect genus *replaces Oncopeltus*. The specimens of this which have been examined thus far have been entirely negative for any flagellate, though infected with Sporozoa both intracellularly and extracellularly in the salivary glands. This coincidence of the range of the milkweed flagellate with the range of *Oncopeltus fasciatus* (Dall.) suggests that the presence of the flagellate is dependent on the presence of the insect. Moreover *Oncopeltus* feeds characteristically on the pods and flowers of the plant, and since plants bearing seed are the ones to which the infection is practically limited it seems even more probable that *Oncopeltus* is the host. Another bit of circumstantial evidence is gained from the study of the morphology of the flagellates from the insect and from the plant. The plant flagellates are characterized by a twist in their ribbon-like bodies. This is rare in insect flagellates, but is found in the case of the parasites of *Oncopeltus*. The insect feeds on latex. The infected plants have swarms of flagellates in *their* latex in every portion of the upper parts of the plants. Thus the insect could easily become infected from his feeding. In the insect the histological studies about to be described have shown swarms of flagellates in definite lobes of the thoracic salivary gland. The secretion of this gland is led by a simple duct directly to the mouth parts during the process of feeding, so that there should be no great mechanical difficulty in the transfer of the insect's flagellates to the interior of the latex ducts of the plant.

It is natural then that in spite of the difficulties which have been experienced up to this time in attempting to obtain plant infections from the insects in regions where plant infections are not known in the field, efforts to establish the relation of the

plant and insect flagellates by histological studies have been made. The results have been negative so far as obtaining definite proof of the identity of the two forms is concerned, but such interesting observations have been made during the study that they will be described here.

The first insects to be sectioned and stained were collected during 1923. At first the flagellates were overlooked, and that for two very good reasons. The principal search was made for them in the intestinal tract, where they do not occur in my material, if indeed they ever occur there. And the salivary gland forms, stained as carefully as they may be, never stand out with the clearness by which those in latex smears are characterized. For the process of drying and staining with Wright's stain, though open to the objection that the nuclear detail is lost, gives bright sharp pictures of the organisms, usually making them much darker than the background. This is far from the case with wet-fixed material, sectioned, and stained even with so good a stain as iron hæmatoxylin. By this process the nucleus may be stained with all the desired sharpness, but the background of salivary secretion retains the stain far more than does the flagellate's cytoplasm. So that usually one sees the body of the flagellate only as an unstained area surrounding the nucleus. The wall of the salivary gland also retains the stain so tenaciously that it must usually be left very black that the internal structure of the flagellate may be seen at its best. This makes the whole field very dark, with the object to be examined exceedingly delicate and lightly stained. When these first specimens of *Oncopeltus* were examined a second time for a different purpose, clusters of the flagellates were noted by chance on the wall of the gland. The slide under observation at the time was stained with a mixture of aniline dyes, but the finding was at once confirmed by iron hæmatoxylin slides of the same material.

During 1924 a quantity of material was obtained for sectioning to discover the extent of the infection among the insects during the late season when the plants were becoming more and more widely infected, to determine with certainty that the organ in question was really the salivary gland of the insect and not a salivary receptacle or some other organ, to see whether the

organism present had more than one stage in its life-history in the insect, and to find exactly its relation to the salivary gland tissues and secretion.

The study of the morphology of the gland in which the flagellates occurred required complete series of serial sections of three insects. The gland of one of these was reconstructed section by section, for the lobes of the gland cannot be readily visualized from the separate sections. Sometimes but one or two show, at other times there appear to be five or six lobes because of the inclination of the section and the twists in the organ itself.

## Histology of the Salivary Gland.

The salivary gland nearly fills the dorsal half of the thorax. Its efferent duct leads away from the point at which the three lobes come together, first running back toward the abdomen immediately under the gland, then turning and running forward. It dips beneath the œsophagus near its junction with the proventriculus, and beneath the ventral chain of nerve ganglia between the sub-œsophageal ganglion and the ganglion immediately posterior to it. From this point the duct runs forward to the mouthparts where its secretion enters the pump or syringe and the hypopharynx.

It was interesting to note that the three parts of the gland could be distinguished readily in iron hæmatoxylin eosin sections by the character of the secretion in each, in spite of the fact that the outline of each might be most deceptively placed according to the plane of the section. The anterior lobe contained frothy material staining pink with eosin. The ventral lobe was distended with a smooth or very slightly granular substance which also stained pink with eosin. It was thus easy to distinguish these two by the consistencies of their contents. The dorsal lobe was remarkable in that its fluid retained some of the iron hæmatoxylin stain and in addition picked up eosin eagerly, nearly attaining the brilliant orange red color characteristic of blood corpuscles in tissues stained for example with Delafield's (not Heidenhain's) hæmatoxylin and eosin.

## Localization in Dorsal and Anterior Lobes.

It was in the third or dorsal lobe that the flagellates were really abundant. There they were often massed like a new tissue lining the entire lumen of the gland. The dark stain which the fluid took made it difficult to study the internal structure of the organisms. Yet they were so abundant that some were usually favorably located for observation. The flagellates lined the dorsal lobe most heavily near its posterior extremity. Nearer the efferent duct the organisms were often in groups or clusters, isolated a little from the rest of the mass, but closely bunched together with flagella attached to the wall.

In the anterior, foam-filled lobe there were a few flagellates always when they were present in the dorsal lobe. In the posterior lobe there were none, no matter how heavily the insect was parasitized.

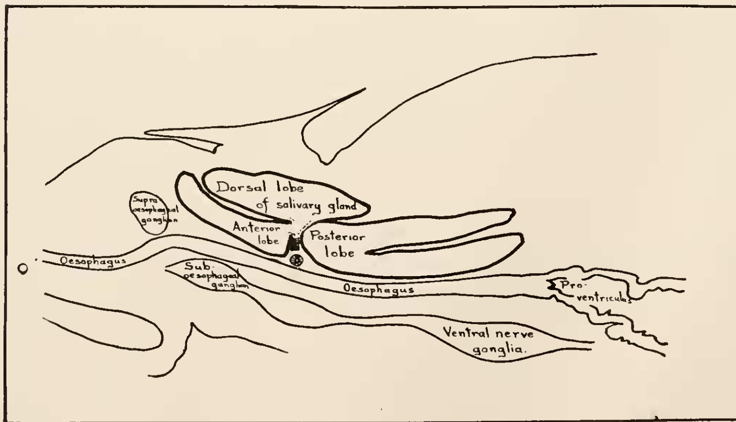


Fig. 3. Portion of a longitudinal section through *Oncopeltus*, showing the relative positions of the salivary gland and other thoracic organs. The three lobes of the gland come together in this particular section and the point of entrance of the salivary duct is shown.

That the fluids from the three lobes intermingled slightly at the common point of contact where the three fluids drained into the same efferent duct was indicated by the mixed consistencies and colors just at this exit point. In the smooth orange-red fluid a cloud of pink was always seen to have penetrated for a

short distance. In the two pink portions, granular and frothy, red clouds were also visible just at the same point.

Until the gland had been reconstructed by drawing each section on blotting paper, cutting out and pasting together the individual sections, and coating the whole model with a beeswax and paraffin mixture, it was impossible to get an adequate idea of the complete gland. The lobes were so complicated in some planes of the section that it was not certain that no salivary reservoir entered the question. As the work was completed, however, it became evident that the structure in which the flagellates were located was a three-lobed thoracic salivary gland, with a single common exit. A diagram of the longitudinal section through the opening of the three lobes is to be seen in Fig. 3.

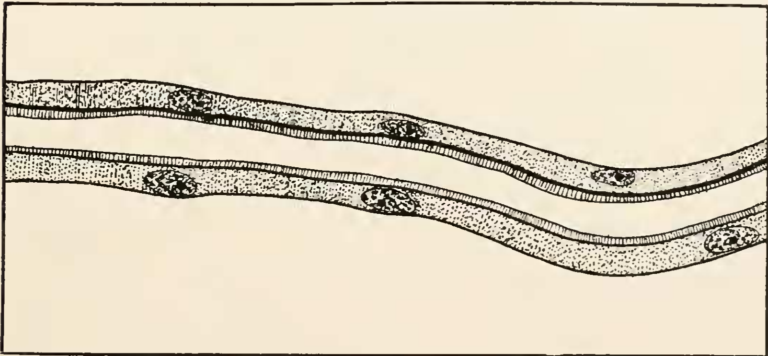

FIG. 4. A portion of the efferent duct of the salivary gland of *Oncopeltus*.

The cells of the duct are characteristic and interesting and with the secreting cells of the gland are so readily recognizable that no difficulty arises in identifying them even in sections which show but a tiny fragment of the salivary apparatus.

The duct is lined with a single layer of cells the nuclei of which are sometimes branched. A portion of the duct is represented in Fig. 4. A similar detailed drawing of the cells of the wall of the salivary gland is shown in Fig. 5. The glands are commonly distended with fluid. This escapes gradually, as required, through the efferent duct.

With the recognition of this organ as a thoracic salivary gland

one of the objects of the histological examination of the insects was accomplished.

It was interesting to note that the flagellates always developed in the dorsal lobe most abundantly. A few penetrated the anterior, but none the ventral lobe. Evidently the differences in the compositions of the salivary secretions of the three lobes, indicated at once by the different staining reactions of the three portions, had also a significant effect on the organisms, inducing them to inhabit one portion more than others, and excluding them from the ventral lobe.

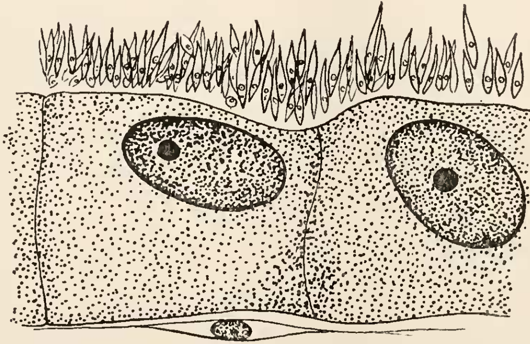

FIG. 5. A portion of the wall of the dorsal lobe of the salivary gland of an infected *Oncopeltus*. The nuclei of the gland cells are gigantic in comparison with the minute nuclei of the flagellates.

In my material no intestinal forms were found. This is remarkable when one considers that the type species of the genus *Herpetomonas* is a strictly intestinal flagellate in the common housefly. Salivary gland forms are the exception rather than the rule among Herpetomonads, and it would be expected that intestinal forms of the flagellate would be more noticeable than the gland forms. But if the flagellates of *Oncopeltus fasciatus* (Dall.) ever show intestinal forms I have not been able to secure the proper material to demonstrate them.

In addition to the complete series of sections of three individuals of *Oncopeltus fasciatus* (Dall.), a group of ten of the same species was sectioned and studied by choosing representative sections for staining and for examination. Sixteen individuals of *Lygæus kalmii* from Massachusetts were treated in the same way.

The object of using *Lygæus* was this: the limit of the range of *Oncopeltus fasciatus* coincides with the limit of the range of the plant flagellate, *Herpetomonas elmassiani* (Migone). But *Lygæus* is a very closely related insect, replacing *Oncopeltus* in the north where the flagellates are not found. It was desired to know whether it also had flagellates in its salivary glands. The thirteen specimens of *Oncopeltus*, which came from Maryland in September, 1924, at a time when the spread of plant flagellates was going on rapidly, were all infected with the exception of three individuals, which were rather young nymphs. The sixteen specimens of *Lygæus* were all negative for flagellates, both in the intestinal tract and in the salivary gland.

In the *Lygæus* examined there *were* infections with sporozoa in the glands, curiously enough in the same lobes frequented by the flagellates of *Oncopeltus*. One of the species present in *Lygæus* was occasionally seen also in *Oncopeltus* along with the flagellates. But in *Lygæus* the infections were much heavier, and decidedly destructive to the gland cells which were penetrated by intracellular stages and often rendered useless for secretion by the growth of the parasites and the consequent death of the cells.

The absence of flagellates in sixteen specimens of *Lygæus* collected at the end of the season was strikingly in contrast with the presence of large numbers of flagellates in the glands of ten of the thirteen *Oncopeltus* sectioned. If *Oncopeltus* is responsible for the spread of the milkweed flagellate it is no wonder that the spread is very rapid in September and early October when so large a percentage of the then rapidly multiplying insects are positive.

Of the original problems for the solution of which the histological work on *Oncopeltus* was carried out, but one remains for consideration. Does the flagellate of *Oncopeltus* have more than one stage of its life history in the insect? It seemed likely at first that there would be developmental stages of the herpetomonad in the intestine. But no colonization of the intestinal tract was indicated by any of my material. All the organisms were in the glands. The question then arose as to the exact position of the parabasal body in the forms colonizing the walls of the salivary gland. Careful study showed that in all cases

in which the relative positions of nucleus and parabasal body could be definitely determined, the organism was constantly a herpetomonad. Smears of the insect flagellate confirmed this, for in them the position of the organelles could be determined in every single case far more readily than in sections of the gland. This question is not quite safely settled, for at some other season of the year the evidence might differ from that which I have been able to gather, but with the knowledge of the exact part of the insect parasitized, the dorsal and anterior thirds of the thoracic salivary gland, and the extent of infection during the season at which the insects are busily feeding on the infected plants, it will be easier to work on the questions concerned with the insect host of the flagellate.

## Summary.

Histological studies of the milkweed host of the flagellate *Herpetomonas elmassiani* (Migone) showed that the organisms were confined to the latex system, in which they were intracellular but not intracytoplasmic. The latex is secreted into the general cell vacuole of the latex duct, and it is in this that the organisms were found. No other cells or parts of cells were found to be penetrated.

During the early part of the summer one or a very few latex cells in a plant were sometimes infected, for in *Asclepias* the original latex cells of the embryo never fuse. Because of this condition occasional localized infections appeared, in which a few leaves of the infected plant were found to be free from organisms.

The flagellates of *Oncopeltus fasciatus* (Dall.), a red and black hemipterous insect suspected of being the insect host of *H. elmassiani* (Migone), were found to inhabit the three-lobed thoracic salivary gland. In the gland these were definitely localized, colonizing only the dorsal and anterior lobes.

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# HEAT PRODUCTION BY THE EGGS OF *ARBACIA PUNCTULATA* DURING FERTILIZATION AND EARLY CLEAVAGE.

CHARLES G. ROGERS AND KENNETH S. COLE.[1]

The general problem of development has in it so much of the unexplained that any attempt to add to our information by attacks from new directions may seem warranted. The work here reported was first undertaken several years ago, but was not carried to a successful issue until this past summer because of experimental difficulties unforeseen at the start.

The work was originally undertaken in an attempt to check the work of Warburg and of Loeb and Wastenys concerning the oxygen consumption of eggs before and after fertilization. It will be recalled that these investigators had found that immediately after fertilization there occurs a remarkable increase in the rate of oxygen consumption, amounting to 4 to 6 times the amount used before fertilization. It is to be assumed that there is also a corresponding increase in the carbon dioxide production of the eggs. This latter would, of course, be exceedingly difficult to measure in the case of marine eggs. If there is any considerable increase in oxygen consumption following fertilization there should be a corresponding increase in the amount of heat produced by the eggs as a result of the oxidation process. The question to be faced was whether, with the facilities at our command, we would be able to make a series of measurements which would bear examination by a physicist. Preliminary tests made in 1920 and 1921 indicated that the production of heat by fertilized eggs was a measurable quantity. These tests, observed in the latter year by a group of physicists who were visiting the Marine Laboratory, proved to be sufficiently encouraging to warrant further expenditures of time and money.

[1] Contribution from the Zoölogical Laboratory of Oberlin College, and from the Marine Biological Laboratory, Woods Hole, Mass. To the Director and other officials of the Marine Biological Laboratory we express our thanks for many courtesies extended to us.

Since this investigation was first projected others have made studies of a somewhat similar character. Myerhof (6) measured the heat production of segmenting Echinoderm eggs by means of a Beckmann thermometer, using a large vacuum flask for his calorimeter. Certain irregularities that occur in his curves may be due to experimental error, but suggest changes in the rate of heat production at different stages of development. Since then Shearer (7) has carried out similar measurements in connection with his work on the oxidation processes of Echinoderm eggs and finds that the rate of heat production after fertilization is constant for at least ten or twelve hours. He makes the statement that readings were taken at fairly frequent intervals at the commencement of the experiment, and at intervals of several hours after that. In view of this statement it seemed advisable to us to repeat the work making frequent readings and using methods of higher precision.

## Possible Sources of Error.

In an investigation involving the measurement of such slight temperature changes as are expected here care must be taken to foresee and provide against all possible heat transfers into or out from the experimental flasks or to know the magnitude of such transfers. In any event such heat losses or gains should be small as compared with the total heat production which it is desired to measure. In our experiments the following possible channels of heat transfer existed, and were checked: Conduction to or from the water in the flasks

(1) by the air in the mouth of the flask,
(2) by means of the glass forming the neck of the flask,
(3) along the main thermopile,
(4) along the secondary thermopile,
(5) along the tubes of the stirring apparatus,
(6) along the fertilizing tube,
(7) by changes in the temperature of the water surrounding the flasks.

There are also possibilities of error arising from lack of care in controlling the conditions environing the electrical apparatus. Among these may be mentioned:

1. Mechanical jarring of the galvanometer used in making the measurements.
2. The temperature of the room must be maintained as nearly constant as possible during the course of an experiment. It was found to be especially necessary to avoid the possibility of drafts of air striking the apparatus.
3. It was found necessary to shield the electrical apparatus from stray electrical currents. This was found to be of the greatest importance following a severe electrical storm.
4. It was found wise to avoid all stress and strain in the wires of the thermopiles, such as might be caused by too much bending of the wires, or placing tension upon them.

It was found possible to obviate much of the possibility of the errors of the first group by using a water cap, designed so as to provide a current of water coursing continuously over the experimental flask as well as around it.

## Apparatus and Methods of Work.

The general method employed was that of the micro-calorimeter, developed by Hill in his work upon muscle. Fig. 1 shows the experimental set-up used. This method has one serious disadvantage. Since corrections for heat loss are dependent upon the temperature difference and the time, long runs can not be made, since these corrections soon become a very large part of the result. In this work the corrections could be kept less than 15 per cent. of the total temperature change during a period of two and a half or three hours. Two straight sided commercial vacuum flasks of about 75 cc. capacity were used, (I.) containing 50 cc. of the egg suspension, and (II.) an equal quantity of water. These flasks had been especially exhausted through the kindness of Dr. W. R. Whitney of the Research Laboratory of the General Electric Company. When flask (I.) contained 50 cc. of water it had a heat loss of 16 calories per hour per degree difference in temperature between the interior and the exterior. The flasks used by Hill (2) had a loss of about 12 calories per hour for 250 cc. and those used by Shearer (7) about 19 calories per hour for 800 cc. This small loss for a flask of such small capacity is evidence of the great value

of very careful evacuation. The flasks were submerged in running sea water to within a short distance of their tops, and a water cap (*d*) made according to a design by White (9) was

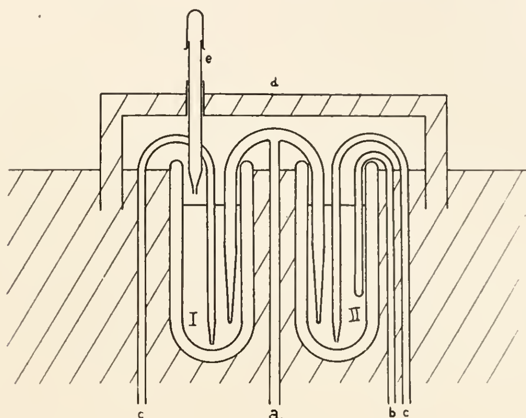


FIG. I. Diagram of experimental set-up. I. and II., Vacuum flasks. *a*, main thermopile. *b*, auxiliary thermopile. *c*, *c*, stirring tubes. *d*, water cap. *e*, sperm pipette.

placed over them. A pipette (*e*) projected through the cap and was arranged so that "dry sperm" could be held in it, and then mixed with the contents of flask (I.) whenever desired. A continuous flow of water was maintained through this cap so that the whole formed a uniform temperature enclosure. The sea water temperature remained fairly constant except for periods of about four hours after sunrise and sunset. The variation of temperature never exceeded 0.001° C. during the period occupied by an experiment.

## Stirring Apparatus.

It has been noted by many investigators that eggs of Echinoderms must not be heaped upon each other if normal development is to take place. In our work it was desirable to use as large a number of eggs as possible in order to get the largest possible temperature change. It was therefore necessary to devise some method of stirring the eggs which would render it impossible for them to settle to the bottom of the flask and remain there for

any considerable length of time. By the more constant stirring the eggs would at all times be able to get their needed supply of oxygen and to get rid of carbon dioxide. Work previously done by one of us (R.) had shown that stirring the water in the experimental flasks once every two or three minutes by means of an ordinary pipette was sufficient to allow the eggs to go through a normal cleavage. Such stirring was not sufficient to ensure that the water in the flask would be of uniform temperature throughout. It seemed necessary, therefore, to devise some automatic stirring device which would keep the water thoroughly stirred at all times during the course of an experiment. As a matter of interest it was found during the course of the experimentation that a failure of the stirring apparatus for as much as three minutes could be detected by a marked variation in the galvanometer readings.

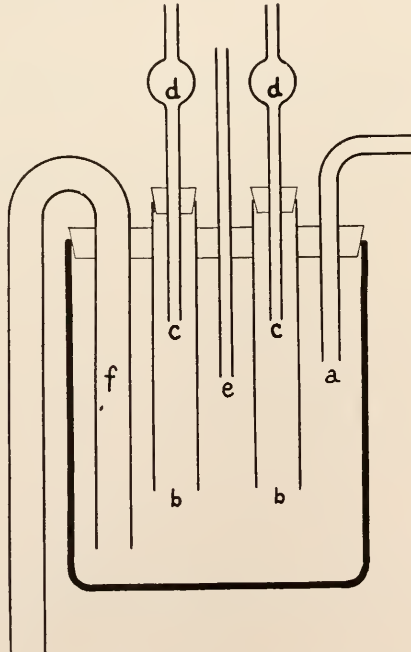


FIG. 2. Stirring apparatus. Details described in text.

The stirring was accomplished as follows: Saturated air was bubbled from the jets (*c*, *c*) at the bottoms of the flasks, by

means of the automatic intermittent siphon device shown in Fig. 2. A steady stream of water flowed in at (*a*) and as the water level rose in the bottle, air was trapped in (*b*, *b*) and forced out through (*d*, *d*). When the water reached the lower end of (*e*) it rose in the tubes, but not in the bottle (since the stopper was air tight) until (*f*) was filled beyond the bend. Then (*f*) acted as a siphon and emptied the bottle, allowing (*b*, *b*) to drain, and finally draining itself. Then the process started over again. Short pieces of rubber tubing cut at an angle were slipped over both ends of (*f*) and the lower ends of (*b*, *b*) to help in breaking the meniscus and draining these tubes. The quantity of air sent over each time could be varied by adjusting the height of the small tubes (*c*, *c*) in (*b*, *b*), and the period of the apparatus could be changed by raising or lowering (*e*). The small bulbs (*d*, *d*) prevented drops of water from being forced over into the wash bottles. These wash bottles had inlet tubes of small cross section, and were submerged in the same bath with the flasks. They served to complete the saturation of the air, if it were not already saturated, to bring it to the temperature of the bath before it went into the flasks through (*c*, *c*), and to act as a trap, preventing the suction of liquid out of the flasks through (*c*, *c*) when the stirrer bottle was emptying. In this work 7 cc. of air was sent through each flask every fourteen seconds. During a six-day continuous run, this apparatus did not vary from its average period by more than 2/5 second, always delivering the same and equal quantities of air to the flasks.

### Electrical Equipment.

The electrical equipment used in these experiments consisted of the main and secondary thermopiles, galvanometer, potentiometer, storage battery cells, Weston Standard Cell, switches, etc. The main thermopile (*a*, Fig. 1) was composed of twenty pairs of copper-constantan junctions, and gave 733 microvolts per degree Centigrade, by direct calibration. (The constantan wire used was that manufactured by the Driver-Harris Wire Company, and sold under the trade name "Advance.") The reason for the unexpectedly low value of the thermopile was not found. The junctions were enclosed in thin glass tubes

filled with naphthalene and the lag of the couple was less than twelve seconds. The auxiliary or secondary thermopile (*b*) had five pairs of junctions and gave the temperature difference between flask II. and the surrounding bath. The e.m.f. developed by the couples was measured by a "White" potentiometer. This instrument, manufactured by Leeds and Northrup, gives dial readings by single microvolts and is so arranged that the resistance in the galvanometer circuit remains constant. This fact makes it possible to read fractions of a microvolt with the galvanometer. In this work one microvolt gave a deflection of 18.5 mm. on the galvanometer scale, so there were about 13,550 mm. per degree. With the high magnification telescope used it was easily possible to estimate fifths of a millimeter on the galvanometer scale. The galvanometer was of the D'Arsonval type, also manufactured by Leeds and Northrup. Its resistance was 13 ohms, period 5 seconds, and sensitivity $10^{-8}$ amperes per cm. with the scale a little over three meters distant. The total resistance of the galvanometer circuit was 53 ohms, which was an ohm more than the critical damping resistance. In spite of this fact there were never any oscillations, even when the sperm were introduced into the experimental flask,—only what seemed to be a very steady and somewhat rapid rise. The galvanometer circuit was shielded as far as possible, as suggested by White (10) to prevent the entrance of leakage currents into the circuit. It was, however, impossible to shield the thermopiles effectively since their leads were carried through the running sea water and very strange and erratic e.m.f.s were introduced into the circuit if the shielding system for the potentiometer and galvanometer had anything to do with the salt water or any of the piping in the room. Care had to be taken not to use for connections any wire that had been unduly bent or in any other way maltreated so as to destroy its homogeneity. On the whole the electrical apparatus worked very satisfactorily.

It was our original plan to use Hill's ingenious application of vacuum flasks to the twin calorimetric method (2) but it was found that for our small flasks the heat loss did not remain even approximately constant as the volume of the contents was varied. It was also impossible to obtain accurate values for the

conduction coefficients from cooling curves for large intervals, since Newton's law of cooling applies to small and constant temperature differences, *i.e.*, equilibrium conditions. This requirement could not be met since there was not available any current measuring device of sufficient accuracy to allow the use of electrical heating. It was necessary therefore, to get the cooling corrections under the actual experimental conditions. Following White (9)

Let $\theta_1$ = temperature of flask (1),
$\theta_2$ = temperature of flask (2),
$\theta_3$ = temperature of external bath.

The temperature coefficient of conduction for the flask is defined as the temperature change between the inside and the outside of the flask in unit time when the temperature difference between the inside and the outside is unity. This may be written in mathematical form for flask (I.), $K_1 =$ ; and for flask (II.), $K_2 =$ .

$$K_1 = \frac{1}{\theta_1 - \theta_3} \cdot \frac{d\theta_1}{dt},$$

$$K_2 = \frac{1}{\theta_2 - \theta_3} \cdot \frac{d\theta_1}{dt}.$$

Similarly, for the heat conduction between the flasks along the thermopile,

$$k_1 = \frac{1}{\theta_1 - \theta_2} \cdot \frac{d\theta_2}{dt},$$

$$k_2 = \frac{1}{\theta_2 - \theta_1} \cdot \frac{d\theta_2}{dt}.$$

Also let $w_1$ and $w_2$ equal the temperature changes due to stirring and evaporation. Then considering temperature changes where there is no liberation of heat in either flask

$$\frac{d(\theta_1 - \theta_2)}{dt} = K_1(\theta_1 - \theta_3) - K_2(\theta_2 - \theta_3) + k_1(\theta_1 - \theta_2) - k_2(\theta_2 - \theta_1) + w_1 - w_2 \quad (1)$$

$$= (K_1 + k_1 + k_2)(\theta_1 - \theta_2) + (K_1 - K_2)(\theta_2 - \theta_3) + w_1 - w_2.$$

$\theta_1 - \theta_2$ is proportional to $\theta_a$, the e.m.f. of the main thermopile,
$\theta_2 - \theta_3$ is proportional to $\theta_b$, the e.m.f. of the auxiliary thermopile.

So we may write

$$\frac{d\theta_a}{dt} = K_a\theta_a + K_b\theta_b, \tag{2}$$

since both theoretically and experimentally, $w_1 - w_2 = 0$ in (1). If the quantity of heat $H$ is liberated in (1) and the heat capacity (water plus the water equivalent of the flask) is $c$, then

$$\frac{d\theta_a}{dt} = \frac{1}{c}\frac{dH}{dt} - K_a\theta_a - K_b\theta_b$$

and

$$\begin{aligned} H &= c\left\{ \int_{\theta_0}^{\theta_a} d\theta + K_a \int_{\theta}^{t} \theta_a dt + K_b \int_{\theta}^{t} \theta_b dt \right\} \\ &= c(\theta_a - \theta_b) + c\left\{ K_a \int_{0}^{t} \theta_a dt + K_b \int_{0}^{t} \theta_b dt \right\}, \end{aligned} \tag{3}$$

where $\theta_0$ is the value of $\theta_a$ when $t = 0$.

Independent runs were made to determine the values of $K_a$ and $K_b$. With $\theta_b$ small as compared to $\theta_a$, $d\theta_a/dt$ was determined over the range for the values of $\theta_a$ used and found to be linear in $\theta_a$. The same was done for $\theta_b$ with $\theta_a$ small. With 50 cc. in both flasks the values obtained were

$K_a$ = .0046 microvolt per minute per microvolt difference,
$K_b$ = .0014 microvolt per minute per microvolt difference.

The water equivalent of the flasks was found to be 8 cc. so that $c = 58$ cc.

## A Typical Experiment.

The preparations for an experimental determination of the heat production of the eggs of *Arbacia* involved a variety of considerations not usual in ordinary experimental work in zoölogy. By careful tests the running sea water of the laboratory had been found to be the most satisfactory form of available thermostat, the temperature of the water changing only very slightly during any experimental period—usually only in thousandths of a degree. Care was, therefore, taken to have all glassware and implements used at the temperature of the sea water. Flasks, pipettes, graduates, beakers, finger-bowls, dissecting instruments, wash bottles of the stirring apparatus, thermopiles, water-cap and the animals to be used were all left in running sea water of uniform temperature for some time before

the beginning of the experiment. Whenever possible all of the eggs used in an experiment were taken from a single female. These were allowed to stand for a few minutes in a finger bowl in a little more than 50 cc. of sea water. The finger bowl was floated in running sea water. Remnants of the ovary and other debris were removed from the finger bowl by forceps or pipette. After a few minutes the eggs were stirred in the water so as to be evenly distributed throughout the whole mass of water and exactly 50 cc. of the suspension was transferred to flask (I.) by means of a volumetric pipette. Also 50 cc. of sea water were placed in flask (II.). The temperature of flask (II.) was then made enough higher than that of flask (I.) so that at the end of the run it would be about as much below (I.) as it was above at the beginning. This made the value of the integral involving $\theta_a$ approximately zero and kept the maximum value of the corrections low. The water-cap with a drop of dry sperm in the fertilizing tube was then put into place, the cap filled with water and the readings begun. The starting of the experiment and the making of the observations and recording them occupied the full time of two persons. Readings of the main thermopile, $\theta_a$, were made every 60 seconds, and of the auxiliary thermopile, $\theta_b$, every five minutes, for a period of from two and a half to three hours. Experiments were in only a few cases continued beyond the three hour limit. When steady conditions had been reached and enough readings had been taken so that the heat production of the unfertilized eggs could be determined, the pipette was lowered until its tip was below the surface of the egg suspension, the sperm washed out and stirred into the suspension, and the pipette raised again. This operation seldom caused an irregularity of more than 0.0005° C. At the same time a sample of the same batch of eggs was fertilized in a finger bowl, and kept surrounded by running sea water. These eggs were examined from time to time. The galvanometer zero and the storage cells for the potentiometer were checked frequently. At the end of the run the average diameter of the eggs was measured, in addition to the usual data on the percentage of fertilization and development. The volume of the single egg computed from the average value of the diameter, and the total volume of eggs

used was determined by centrifuging the suspension. After the centrifuging, the eggs were deformed so that their volume represented almost all of the volume measured; thus it was possible

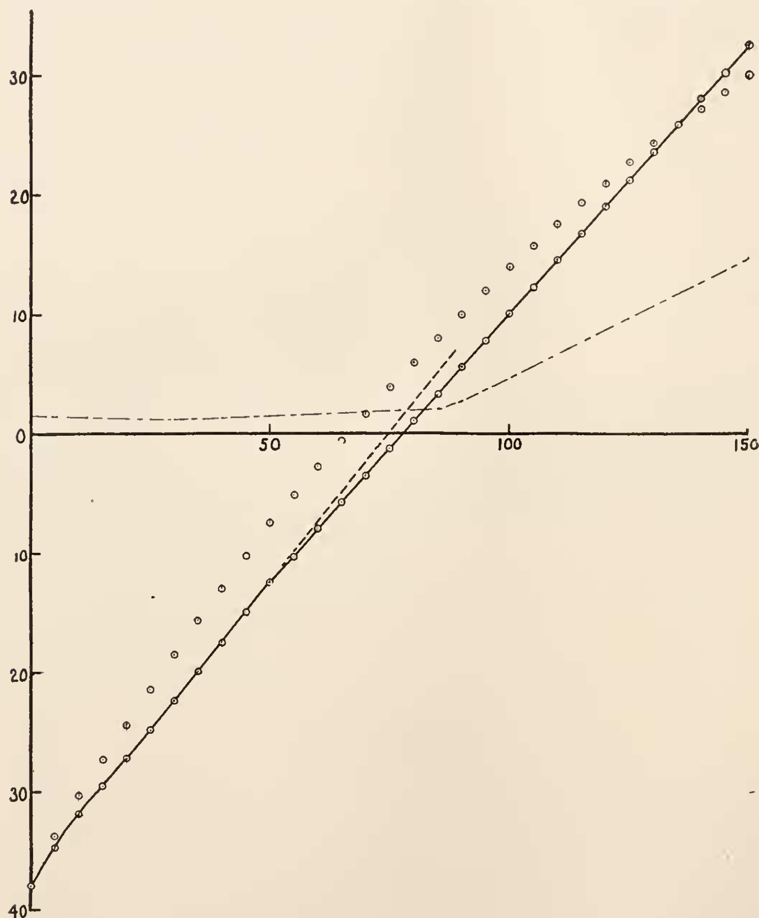


FIG. 3. Typical experimental curve. Heat production of *Arbacia* eggs. Abscissæ, time in minutes after fertilization. Ordinates, temperature differences in micro-volts. $1 \mu v = 0.001364°$ C. ⊙ $\theta_a$ observed values. — – — $\theta_b$ observed values. —⊙— corrected curve. — — — projection of corrected curve of 20 to 50 minute period beyond the 50 minute position.

to obtain an estimate of the total number of eggs used. This method of counting was checked against a dilution method, similar to that employed for counting blood corpuscles, and it

was found that the agreement was very close, and that the eggs showed less variation than did the number of eggs in the fractions counted.

The course of a typical run is shown in Fig. 3. In it the average diameter of the eggs was 74 microns, and the total

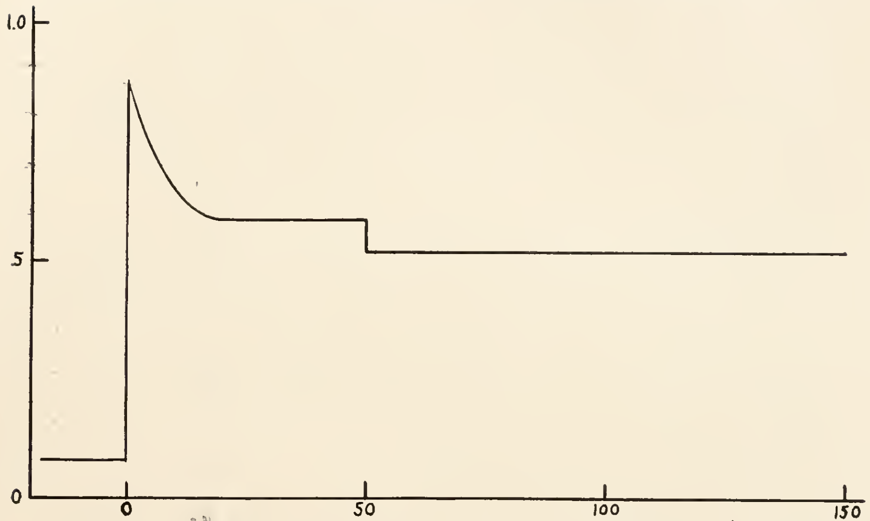


FIG. 4. Rate of heat production, *Arbacia* eggs. Abscissæ, time in minutes after fertilization. Ordinates, rate of heat production in calories per hour per million eggs.

volume was 0.82 cc. The total number of eggs was, therefore, about 3.9 millions. About 96 per cent. of these eggs were fertilized and of those fertilized about 85 per cent. were in the eight cell stage, and the rest in a late four cell stage and going into the eight cell stage so rapidly that an accurate percentage could not be obtained, when the experiment was concluded. In Fig. 3 every fifth reading is plotted. The corrected curve was obtained by taking approximate values of the integrals of equation (3) for five minutes intervals. The greatest variation in the different runs is in the behavior during the first twenty minutes after fertilization. This is probably due to the fact that the amount of sperm could not be made proportional to the number of eggs, and that the heat production of the sperm is not negligible during this period, as will be shown later. In this

respect the run shown in Fig. 3 shows a marked variation from the average.

The average of seven runs is shown in Fig. 4, which gives the approximate *rate* of heat production. The rate of heat production per million unfertilized eggs is about 0.08 calories per hour, while for the fertilized eggs after they have gone into the two cells stage it is about 0.52 calories per hour.[1] Both of these values are higher than those obtained by Shearer and by Myerhof with other sea urchins, but the ratio of fertilized to unfertilized eggs is the same. It should be pointed out that the results here given must be taken as indicative rather than conclusive and that further painstaking work is necessary.

One feature of the curve shown in Fig. 4 deserves special comment. It will be noted that the greatest rise in temperature, *i.e.*, the greatest period of heat production occurs immediately upon fertilization. This certainly raises again the question as to whether the process of membrane elevation depends upon an oxidative process, set up by the sperm cell when it comes into contact with the surface of the egg.

It must be mentioned here that Loeb (3) had expressed in 1906 the view that the essential feature (or possibly one of the essential features) of the process of fertilization is the increase in the rate of oxidation in the egg, and that this increase is caused by the membrane formation. Both Warburg and Loeb and Wasteneys had shown that the rate of oxidation in the sea-urchin egg is increased from 400 per cent. to 600 per cent. upon the entrance of the spermatozoön—and that membrane formation alone, induced by artificial means, has the same result. There is, therefore, a definite relation existing between membrane formation and increased rate of oxidation. From Warburg's (8) work it also seems likely that the increased oxidation occurs chiefly at the surface of the egg. The fact that the greatest heat production by the egg comes immediately after fertilization seems to us to make it plausible to say that the entrance of the spermatozoön induces a cortical oxidation process, and that this process results in the elevation of the fertilization membrane. The almost explosive character of the heat evolution seems to

[1] This involves an energy liberation of approximately 1 erg per egg per hour.

indicate that the oxidative process is of fundamental importance in the series of fertilization phenomena.

In the only run made upon sperm, ten drops of dry sperm were placed in the pipette and mixed with 50 cc. of sea water after steady conditions had been established. The heat produced can be expressed quite accurately by an equation of the form

$$H = H_0(1 - e^{-bt}),$$

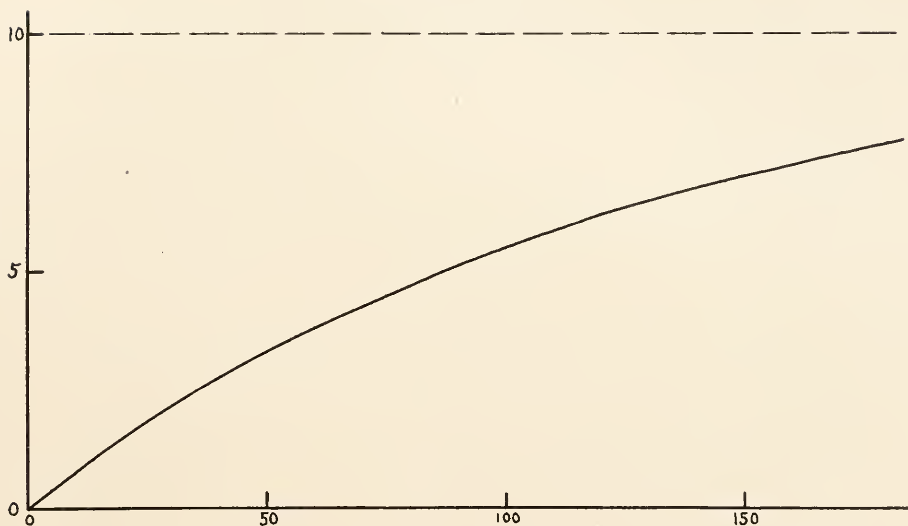


FIG. 5. Heat production of *Arbacia* sperm. Abscissæ, time in minutes after one drop of sperm is placed in 50 cc. sea water. Ordinates, temperature difference in micro-volts.

where $H_0$ is the total amount of heat produced—which, from the work of Cohn (1) is probably a constant for any given amount of sperm, $b$ is probably dependent upon the pH of the water and the temperature, $e$ is the natural base of logarithms, and $t$ is the time after the sperm comes in contact with the water. This equation suggests very much the heat production by an exothermic reaction of the first order. In this case for ten drops of sperm,

$$H_0 = 0.79 \text{ calories},$$
$$b = 0.008 \text{ when } t \text{ is in minutes.}$$

Figure 5 shows the temperature change when one drop of sperm (approximately the amount used) is added to 50 cc. of

sea water. The curve approaches 10 micro-volts as an asymptote, but reaches 50 per cent. of that value in about 90 minutes. From this it will be seen that if there is an excess of sperm it may seriously affect results during the first twenty or thirty minutes.

As has been pointed out this method can not be used over long periods of time with the desired degree of accuracy. It also has the disadvantage of requiring large numbers of eggs, so that the longer they run, the more they tend to fall out of step, and so tend to mask any effect that may be present. This latter difficulty will remain, of course until it is possible to work with a single egg. The apparatus is being redesigned so that it will be possible to follow the heat production of both eggs and sperm under varying conditions over longer periods of time. It is planned to extend the work so as to include other forms.

## Summary.

The heat production of the eggs of *Arbacia punctulata* has been measured before, during, and following fertilization, through development into the eight cell stage. It has been found that the rate of heat production at the instant of fertilization is ten to twelve times that of the unfertilized egg. After fertilization the rate of heat production decreases constantly for twenty minutes, when it reaches about 65 per cent. of the value at fertilization, and remains constant until the first cleavage, at about 50 minutes after fertilization. At the first cleavage the rate drops suddenly by more than 10 per cent., and then remains constant until the eggs are in the eight cell stage, which is as far as the work has been carried. The rate of heat production of the unfertilized eggs was found to be about 0.08 calories per hour per million eggs, and that of the fertilized eggs about 0.52 calories per hour per million eggs after the one cell stage.

An experiment on *Arbacia* sperm indicates that when placed in contact with sea water, its heat production is similar to that of an exothermic chemical reaction of the first order.

The suggestion is offered that the heat evolution occurring immediately upon fertilization is the result of an oxidative process which takes place in the cortex (chiefly) of the egg and which leads to the elevation of the fertilization membrane.

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# CONCERNING THE RELATIVE PHOTOTROPISM OF VESTIGIAL AND WILD TYPE *DROSOPHILA*.

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In a previous investigation upon various phases of the light and gravity reactions of *Drosophila melanogaster*, the present writer concluded among other things, that the mutant race known as vestigial is much less phototropic than is the wild fly.[1] Subsequent to this, however, W. H. Cole, using a somewhat different technique from that of the author, arrived at quite another conclusion.[2] He made his tests in a vertically placed glass tube, with the source of light above it, and under these conditions he found that vestigial flies when attempting to climb the smooth walls of the tube constantly lost their footing, and slipped back. Thus they made poorer records than did winged insects, which, when they slipped, used their wings to catch themselves. This circumstance, according to Cole, entirely accounts for any apparent difference in the phototropic response of these two types of *Drosophila*. The correctness of this interpretation he seems to have proven by lining the glass with thin rice paper which enabled the wingless animals to obtain a secure footing, under which condition they made as good records as did the flies with wings.

It is to be noted that throughout these experiments only tests with a vertically placed tube were made. This was done on the assumption that the negative response to gravity which *Drosophila* gives would be constant, and that, therefore, any additional response would be due solely to the light placed above the tube. That this was apparently true Cole showed by testing the insects when only a very dim red light was present, as well as with lights of varying intensities. Nevertheless, in view of the

[1] McEwen, R. S., "The Reactions to Light and to Gravity in *Drosophila* and its Mutants," *Jour. Exp. Zoöl.*, 25: 49, February, 1918.

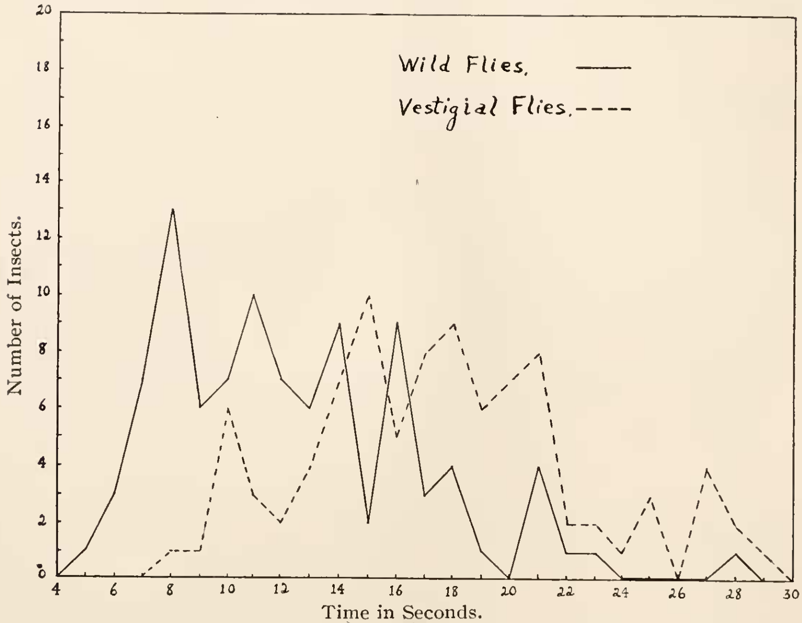
[2] Cole, W. H., "Note on the Relation Between the Photic Stimulus and the Rate of Locomotion in *Drosophila*." *Science*, LV., No. 1434, June 23, 1922.

author's previous results it seemed to him that either this mixing of the two responses, or perhaps some other unrecognized factor, might have produced complications which would account for the discrepancy in results. It was, therefore, determined to repeat Cole's work, using so far as possible his own technique, but with the addition of tests in horizontal tubes. The method employed was as follows:

A number of wild and vestigial flies were kept in bottles containing a plentiful supply of food in which larvæ were working, until five days after hatching. On the eve of the sixth day from ten to fifteen insects of each kind and of the same sex were removed, etherized, and each animal placed in a separate vial with a small amount of food. The next morning each insect was given a preliminary test within its vial, to determine so far as possible its general activity. This was done for the vestigials by turning the glass one end up and then the other four or five times. The five vestigial flies which responded most readily by giving the negative reaction to gravity, and which showed the greatest facility in crawling up the smooth glass of the vials, were then selected for the critical tests. Five wild flies were similarly chosen, except that their reaction to light was used as a criterion of activity, and an effort was made to pick those which reacted by crawling rather than by flight. In this connection it might be thought that the use of light rather than gravity as the stimulus for the winged flies would result in the selection of particularly phototropic animals. However, since virtually all wild type *Drosophila* are decidedly phototropic, the fact that those which only crawled to the light were chosen, would mean that if anything the less phototropic ones were selected. This preliminary work was then immediately followed by the critical tests of the ten flies in a manner to be described below. A similar procedure was continued on successive days until fifty wild males and fifty vestigial males had been tested; the same was then done with fifty females of each kind, making a total of 100 insects of each type. Each day's critical tests involved the following apparatus and manipulation:

Two tubes were used, one with no lining, and the other lined with thin rice paper throughout the portion through which the

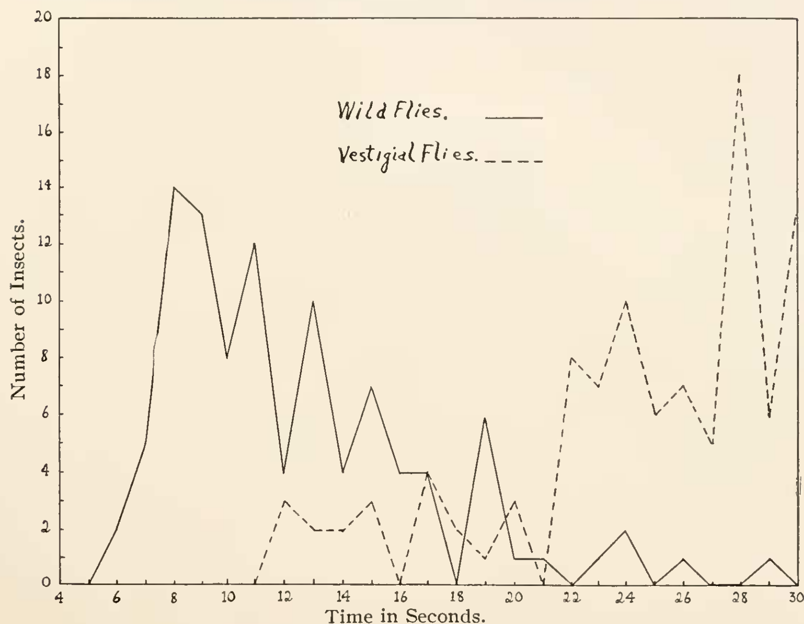
fly was to crawl. Each tube measured 16 mm. in internal diameter, and the length from the end away from the light to the point where the trial terminated was 172 mm., this being identical with the distance employed by Cole. Beyond this point the tube continued for 70 mm. to the end, which was




GRAPH I. The solid line shows the record, corrected for use of wings, of 95 wild flies, 45 males and 50 females, in an unlined vertical tube. The broken line, shows the record, corrected for slipping, of 92 vestigial flies, 45 males and 47 females also in an unlined vertical tube. In each case the time is plotted against the number of insects.

sealed with a piece of cover slip. A 75 watt gas filled lamp on a 110-volt alternating current was used as the source of illumination, and the distance from the end of the tube furthest from the light to the center of the concentrated filament of the lamp was approximately 317 mm. At the point from which the fly started, therefore, it was subject to an intensity of about 1,238 candle meters, an intensity slightly less than the maximum of 1,500 candle meters used by Cole. At the beginning of a test an insect was transferred from its vial to the testing tube by making use of its light or gravity reaction, or when necessary

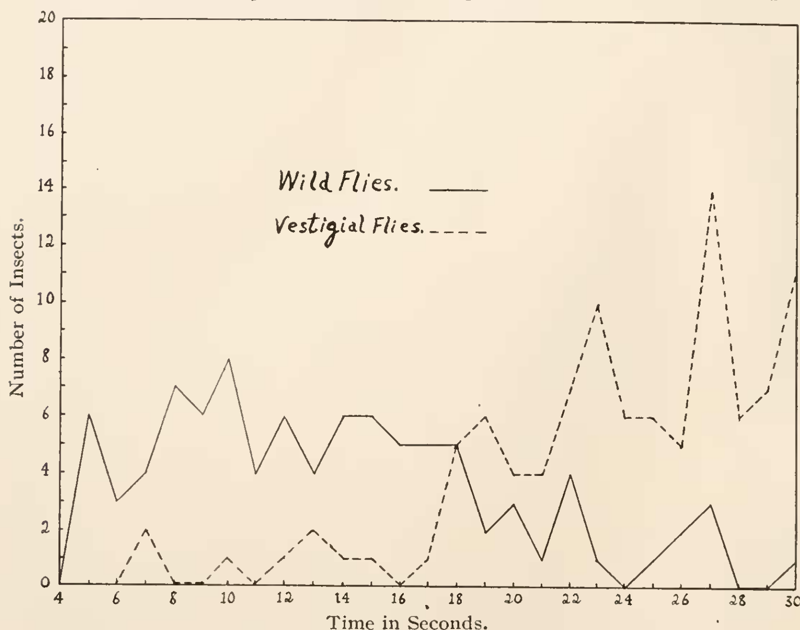
by shaking. The end of the tube was stopped by a cork covered with black paper, and the tube was placed in position. Ten successive trials were given in each test, the time of movement through the distance of 172 mm. being taken by a stop watch. If the passage was not accomplished within 30 seconds, a trial was considered completed, and 30 seconds was recorded as the




GRAPH II. The solid line shows the record of 100 wild flies, 50 males and 50 females, in a lined vertical tube. The broken line shows the record of 100 vestigial flies, 50 males and 50 females, also in a lined vertical tube. In each case the time is plotted against the number of insects.

time made. After each trial the animal was immediately returned to the starting point, if possible by its light or gravity reaction, but frequently by shaking. The latter procedure was made necessary in order that an approximately uniform period (about half a minute) might elapse between each trial. In this manner each of the five flies of a given type was tested in immediate succession, the same tube (either the one lined by paper or the one unlined) being used in the same position (either vertical or horizontal) for each insect. When the test of one group of flies was thus completed, the vials containing the insects were

set aside in a dim light, and the test of the other group begun. In this way each of the two groups were given during the day four tests, so spaced that the time elapsing between the successive tests of any given insect was approximately two hours. Each of the four tests was of course varied as regarded either the type of tube used or its position, the four possibilities involved being:




GRAPH III. The solid line shows the record, corrected for flight, of 93 wild flies, 43 males and 50 females, in an unlined horizontal tube. The broken line shows the record of 100 vestigial flies, 50 males and 50 females, also in an unlined horizontal tube. Only three instances of slipping were noted in the case of the vestigials, and as correction for these would have made no significant difference it is not shown.

the unlined tube horizontal, the unlined tube vertical, the lined tube horizontal, and the lined tube vertical. Furthermore, for the purpose of overcoming any biased effect which might result from always testing the same type of fly in the same condition with respect to time of day and previous tests, the order of the tests was rearranged each day. Under this plan during the course of the experimen'ts each of the two types of insects was tested under almost (though not quite) every possible combination with regard to the above factors. The variations in the

arrangement of the tests thus obtained, though not entirely complete, were felt to be at least amply sufficient to avoid any significant effect from such a source of error. The results obtained by these methods are presented concretely in Table I., and Graphs I., II., and III.

TABLE I.

WILD FLIES.

| Tube Horizontal. | | Tube Vertical. | |
|---|---|---|---|
| Test I.,[1]<br>Lined. | Test II.,<br>Unlined. | Test III.,<br>Lined. | Test IV.,<br>Unlined. |
| Male Female<br>12.6 12.9<br>Combined Average<br>12.8<br>Probable Error<br>.287 | Male Female<br>10.5 15.2<br>Combined Average<br>12.8<br>Probable Error<br>.341 | Male Female<br>11.7 13.9<br>Combined Average<br>12.8<br>Probable Error<br>.31 | Male Female<br>10.8 14.2<br>Combined Average<br>12.5<br>Probable Error<br>.29 |
| | Corrected for Flight<br>Male Female<br>12.1 16<br>Combined Average<br>14.2<br>Probable Error<br>.425 | | Corrected for Flight<br>Male Female<br>11 14.3<br>Combined Average<br>12.7<br>Probable Error<br>.308 |

VESTIGIAL FLIES.

| Tube Horizontal. | | Tube Vertical. | |
|---|---|---|---|
| Test I.,[1]<br>Lined. | Test II.,<br>Unlined. | Test III.,<br>Lined. | Test IV.,<br>Unlined. |
| Male Female<br>24.7 26.1<br>Combined Average<br>25.4<br>Probable Error<br>.25 | Male Female<br>22.9 25.2<br>Combined Average<br>24<br>Probable Error<br>.335 | Male Female<br>24 25<br>Combined Average<br>24.6<br>Probable Error<br>.325 | Male Female<br>18.5 19.1<br>Combined Average<br>18.8<br>Probable Error<br>.348 |
| | | | Corrected for Slipping<br>Male Female<br>17.4 18.5<br>Combined Average<br>18<br>Probable Error<br>.337 |

[1] These numbers are merely for convenience in designating, and do not indicate that the tests were always given in this order (see text).

As regards the mathematical procedures used in obtaining the values shown in the table and graphs the following should be said: The record of each fly in any given test was secured by taking the average of its ten trials in that test. The 100 records of all the individual insects of one type (winged or vestigial) were then in turn averaged for each of the four tests indicated. Both in making the graphs and in computing the probable errors the records of the 100 flies obtained as just described were employed, rather than the results of all their individual trials. This was the essential procedure throughout, but the details were slightly modified in certain cases for these reasons. It was realized shortly after the beginning of the experiments that in view of the question at issue it would be highly desirable to have for comparison averages which had been corrected, so far as possible, for the factor of flight in the case of the winged flies, and for slipping upon the unlined glass in the case of the vestigials. The modifications thus made necessary in the case of the winged animals in the unlined horizontal tube were as follows: Since 7 insects had been tested before observations on this point were begun, the records of these 7 had to be thrown out entirely. In the cases of the remaining 93 flies, wherever necessary new records were computed for each animal by eliminating any of its ten trials in which use of the wings in any manner had occurred. This process involved, as it turned out, 53 insects, with a total of 157 trials, which is an average of 2.9 trials per insect concerned; the highest number which any one animal thus lost was 7. The new records of these 53 flies were then averaged in with the 40 which required no change to obtain the so-called "corrected for flight" average. In the case of the winged flies in the vertical tube, correction was made in the same manner, except that in this case only the records of the first five animals had to be entirely discarded because observation on this point did not begin until after they were tested. In this instance out of the remaining 95 flies the records of 40 had to be corrected, involving 70 trials, an average of 1.75 trials per fly concerned. Again the highest loss suffered by any one animal was 7. The third correction was that for the slipping of vestigial flies in the vertical unlined tube. The same general method was employed as in

the case of flight, a total of 16 animals being affected. Of these the records of 8 had to be eliminated entirely because no effort had been made to distinguish their behavior in individual trials. To the 80 trials thus counted out had to be added 11 trials distributed among the other 8 flies, the highest number lost by any one of the 8 being 3. Thus a total of 92 insects were tested, the records of 8 of them being corrected by the elimination of the trials in which slipping occurred.

It will be noted of course that the probable error in all the determinations is quite high, as the character of the curves would lead one to expect. This is due to the usual erratic behavior of *Drosophila*, which the writer has always found characteristic of this animal. Nevertheless, in view of the very marked differences between the corresponding records of wild and vestigial insects, it is felt that the results are certainly significant. Thus it may be noted that even when the probable errors are multiplied by five, the ranges so obtained do not overlap, except in a single instance. This instance is that of the range for wild flies in the horizontal unlined tube corrected for use of wings, and the range for vestigials in the vertical unlined tube corrected for slipping. Here the upper value for the range of the wild flies just equals the lower value for the range of the vestigials.

The conclusions indicated in the table and graphs may now be summarized thus: (1) The vestigials under every condition are decidedly slower than the wild type; (2) the writer's work fails entirely to confirm Cole's contention that the slipping of the vestigials is responsible for their slower records.

These conclusions come out even more clearly when the data are analyzed in more detail. Thus not only does correction for use of wings by the wild flies make no significant difference, either in the horizontal or vertical records, but correction for slipping in the case of the vestigials likewise produces essentially no effect. Curiously enough indeed, it appears both from the table and the graphs that the vestigials did much better in the vertical tests when the tube was *unlined*, while no very marked difference occurs between their records for the lined and unlined tubes in the horizontal tests. In the case of the wild flies, on

the other hand, there is no significant difference between the records for the lined and unlined tubes in either the horizontal or vertical tests. In this connection it might further be stated that not only does the data based upon timing indicate that slipping is not a significant factor in retarding the vestigials, but that observation of their actual behavior leads to the same conclusion. Thus in the vast majority of cases the writer was unable to see that the vestigial insects had the slightest difficulty in crawling upon the unlined glass. Regarding the matter from yet another aspect it is instructive to note the records for corresponding groups where the only difference involved is the position of the tube. Here it appears that in the case of the wild flies in the unlined tubes the vertical position is slightly superior both as regards the arithmetical average and the mode. In the lined tubes on the other hand, only the modal value for the vertical position is superior. As regards the vestigials, the lined tubes again show only a slight superiority for the vertical records but in the case of the unlined tubes the vertical position is markedly superior, both as to average and mode, *even without the correction for slipping*. This of course is exactly opposite to what one would expect according to Cole's results and interpretation.

Besides this analysis of the data as a whole, it is also pertinent to call attention to certain facts brought out by observation of individual insects. Thus in the case of the horizontal tests of vestigial flies in the unlined tubes, where the behavior of the animals could be perfectly observed, the following feature was noteworthy. There were 14 insects which were noted on the records, not only as having no tendency to slip, but as being unusually active; *i.e.*, they crawled rapidly and constantly about. Yet the average time for these 14 cases was 23.5 seconds, virtually the same as that of the general average for the test. There were likewise 16 vestigials in this test which were recorded as "quite" active, with an average of 24 seconds, and 12 recorded as "fairly" active with an average of 27.4 seconds. Hence the mere activity of these animals does not seem to have materially affected their tendency to move toward the light. The only feasible explanation of such cases would seem to be that such flies are only weakly phototropic. This interpretation is given

further emphasis by one other observation. It was noted in the horizontal tests of vestigial animals that a fly which had reached the light end of the tube would often turn immediately about and crawl quite as rapidly all the way to the dark end. Such a performance, though it would appear very favorably on the record, would obviously not denote any great degree of phototropism. This occurred, however, in at least one of the trials of each of 19 individuals. On the other hand, such action was noted in only two trials of wild type animals. In the case of vertical tests it never occurred with vestigials, but one of the two instances of wild flies took place in such a test. The reason for this single instance is not clear, and in the present state of our knowledge can only be ascribed to the erratic tendency already noted in *Drosophila*. In a similar category, perhaps, must be put the behavior of 9 vestigial insects which, though recorded as active, made relatively poor records in the vertical tests.

How the results and conclusions indicated by Cole can be reconciled with those herein presented it is rather difficult to see. In any event it is perhaps worth while summing up certain outstanding points which the two sets of experiments seem to bring out. In the first place it does not appear that the mixing of the light and gravity reactions, which was suggested as a possible source of error, has any effect upon the main question at issue. Secondly, though also apparently without bearing upon this question, is the fact that Cole's wild type flies in vertical tubes seem to have been much faster than those of the writer. With 1,500 candle meters 50 per cent. of Cole's animals averaged 6.17 seconds and with 750 candle meters 7.6 seconds. Upon the other hand, the best average obtained by the writer was 10.5 seconds, the remainder of them being from two to four seconds slower, under a stimulus of 1,238 candle meters. Such a discrepancy almost causes one to wonder whether there may not have been some fundamental difference in the character of the insects used or in the experimental conditions. Thus, for example, the tests herein recorded were conducted at an average temperature of 24.1 degrees centigrade, and it is known in a general way that the activity of *Drosophila* varies more or less

directly with this factor. Unfortunately Cole gives no record of temperature. Nevertheless, so far as data on this point go, it seems improbable that his work was done at a sufficiently higher temperature to account for the difference in results. In this connection another feature of Cole's work which makes for some uncertainty is the fact that the records for his flies just cited apparently refer to only 50 per cent. of the insects tested. What the other 50 per cent. did might possibly be significant, at least as regards comparative records for the wild type. Lastly, perhaps the most pertinent point to be indicated is this. One conceivable explanation for the poor showing of Cole's vestigial animals in the unlined tube is the fact that presumably they were not previously selected for their ability to crawl on glass, as was the case to a certain extent with those used by the writer. It seems clear, however, that neither this, nor any of the other points mentioned, account at all for the fact that whereas Cole's results showed the vestigials, when given a proper footing, nearly or quite the equals of his winged flies, in the present experiments the wild type completely outclasses the vestigials under all conditions, even when correction has been made for use of wings by the former and slipping by the latter.

# THYROID FEEDING AND SECONDARY SEX CHARACTERS IN RHODE ISLAND RED CHICKS.

HARRY BEAL TORREY AND BENJAMIN HORNING.

In an earlier paper [1] we called attention to the precocious appearance of the second set of rectrices in Brown Leghorn chicks whose daily ration from the fourth week after hatching had included dessicated thyroid. Reference was made also to another case of precocious development of rectrices, namely in Rhode Island Red male chicks,[2] as a result of thyroid feeding. And, correlated with the early appearance of the rectrices in the latter was a failure of the feathers on the neck to differentiate into the hackles characteristic of the control males. The acceleration of rectrices and absence of hackles of the male type combined to give to the plumage of the thyroid-fed males an aspect so strikingly female as to deceive even experienced poultry fanciers. Comb, wattles, carriage of the body and behavior remained, however, characteristically male. The accompanying tables present typical facts and the figures typical birds.

Table I. shows the effect of thyroid feeding in one experiment on the development of the first rectrices in Rhode Island Red chicks. When the latter were four weeks old, they were divided into two lots as indicated in the table. To each bird of one lot a capsule of Armour's desiccated thyroid, containing .2 per cent. I, was given daily. For the first two weeks the dose was 50 mgms.; for the next three weeks, 100 mgms.; for the next three weeks, 150 mgms. The other lot served as a control. All birds were twelve weeks old when recorded.

Figure 1 is from a photograph of the typical bird recorded as No. 1, Table I., at the age of twelve weeks. The rumpless condition is characteristic of normal Rhode Island Red males at this age. It is due to the fact that the first set of rectrices customarily

[1] Torrey and Horning, The Effect of Thyroid Feeding on the Moulting Process and Feather Structure of the Domestic Fowl, Biol. Bull., XLIX, 1925, 275.

[2] Torrey and Horning, Hen Feathering Induced in the Male Fowl by Feeding Thyroid, *Proc. Soc. Exper. Biol. and Med.*, XIX., 1922, 275.

TABLE I.

| Control Birds. | | | | Thyroid-fed Birds. | | | |
|---|---|---|---|---|---|---|---|
| No. | Sex. | Rectrices. | Type. | No. | Sex. | Rectrices. | Type. |
| 1 | Male | 0 | Male | 11 | Male | + | Female |
| 2 | Male | 0 | Male | 12 | Male | + | Female |
| 3 | Female | + | Female | 13 | Female | + | Female |
| 4 | Female | + | Female | 14 | Male | + | Female |
| 5 | Male | 0 | Male | 15 | Male | + | Female |
| 6 | Male | 0 | Male | 16 | Male | + | Female |
| 7 | Female | + | Female | 17 | Female | + | Female |
| 8 | Female | + | Female | 18 | ? | + | Female |
| 9 | Female | + | Female | 19 | Female | + | Female |
| 10 | Male | 0 | Male | 20 | Female | + | Female |

A plus sign indicates the presence of rectrices.

FIG. 1. Normal R. I. R. male (No. 1, Table I.) twelve weeks old. The absence of rectrices is typical at this age.

does not appear. There are exceptions to this rule, but in these cases, the rectrices are small and more or less atypical. There is some variability in the form of the first rectrices in the female, the stoutness and placement of the feathers and the shape of the feather tips being most frequently affected, less often the number. But whatever their form, the rectrices are characteristically absent in the male, present in the female.

Fig. 2. R. I. R. male (No. 12 of Table I.) twelve weeks old, thyroid fed for eight weeks. Rectrices well developed.

In thyroid-fed males, they are characteristically present also, appearing about the time they appear in normal females. Fig. 2 is from a photograph of No. 12, Table I., twelve weeks old. Fig. 3 is another thyroid-fed bird, aged twelve weeks, being No. 15, Table I. In each case the head is typically male, the feathering typically female. Comparison of Figs. 2 and 3 with Figs. 4 and 5

will emphasize these facts. Fig. 4 is from a photograph of No. 4 Table I, a typical control female twelve weeks old. Fig. 5 is from a photograph of No. 13, Table I, a typical thyroid-fed female of the same age.

FIG. 3. R. I. R. male (No. 15 of Table I.) twelve weeks old, thyroid fed for eight weeks. Rectrices well developed.

There was no essential difference between the last two birds at the time the photographs were taken. The conclusion, however, that thyroid feeding does not affect the female plumage at this age must not be hastily drawn. When thyroid-fed chicks are compared with controls at an earlier age, evidence is obtained to show that thyroid feeding accelerates the development of both rectrices and contour feathers in females as well as males.

In Table II. such evidence is summarized. The chicks were 51 days old. Eight days after hatching, thyroid feeding had been begun with 15 mgms. as the initial daily ration, in capsule as

before. This had been increased from time to time, the chicks being fed as much thyroid as they could stand without displaying obvious signs of weakness or distress.

TABLE II.

| Control Birds. | | | | Thyroid-fed Birds. | | | |
|---|---|---|---|---|---|---|---|
| No. | Sex. | Rectrices. | Contours. | No. | Sex. | Rectrices. | Contours. |
| 65 | Female | 1 cm. | Not well out | 81 | Female | ? | Not well out |
| 66 | Female | 0 | " " " | 82 | Female | 7.5 cm. | Well out |
| 67 | Female | 0 | " " " | 83 | Female | 2.5 cm. | " " |
| 68 | Female | 0 | " " " | 84 | Female | 1 cm. | " " |
| 69 | Female | 0 | " " " | 85 | Female | 5 cm. | " " |
| 70 | Female | 0 | " " " | 86 | Female | 2.5 cm. | " " |
| 71 | Female | 0 | " " " | 87 | Female | 2.5 cm. | " " |
| 72 | Female | 0 | " " " | 88 | Female | 0 | " " |
| 73 | Male | 0 | " " " | 89 | Male | 0 | Not well out |
| 74 | Male | 0 | " " " | 90 | Male | 2.5 cm. | Fairly well out |
| 75 | Male | 0 | " " " | 91 | Male | 1 cm. | Well out |
| 76 | Male | 0 | " " " | 92 | Male | 1 cm. | " " |
| 77 | Male | 0 | " " " | 93 | Male | 4 cm. | " " |
| 78 | Male | 0 | " " " | 94 | Male | 2.5 cm. | Not well out |
| 79 | Male | 0 | " " " | 95 | Male | 4.5 cm. | Well out |
| 80 | Male | 0 | " " " | 96 | Male | 4.5 cm. | " " |
| | | | | 97 | Male | 0 | Not well out |

It will be seen on inspection of Table II, that there is considerable variation in the length of the rectrices and the condition of the contour feathers in thyroid-fed birds of both sexes. There are differences, not here recorded, in size and weight also, that are not, however, strictly correlated with differences in the feathering.

FIG. 4. Normal R. I. R. female (No. 4, Table I.) twelve weeks old.

We are aware that such variability is a source of difficulty in the interpretation of results. It is greatest in the first few weeks after hatching, when it is attributable partly to irregularities in nutrition and to differences of vigor referable to other causes. These tend to disappear with age. But the constitutional variability of the Rhode Island Red breed, which we have learned to recognize in the course of our work, remains as a source of error. All of our experiments have been checked in other breeds, notably in Leghorns (Torrey and Horning, 1925) and Campines, where

FIG. 5. R. I. R. female (No. 13, Table I.) twelve weeks old; thyroid fed for eight weeks.

the forcing effect of thyroid feeding on the development of the plumage is clear and definite. In the latter breeds, however, the first rectrices normally develop simultaneously in both sexes.

The variability of Rhode Island Reds led us formerly into a conclusion that proved to be erroneous. In the first series of experiments reported, our five thyroid-fed capons failed to develop rectrices like unaltered thyroid-fed males. Later it was discovered that this result was exceptional. The gonad had, in the male, no bearing on the result.

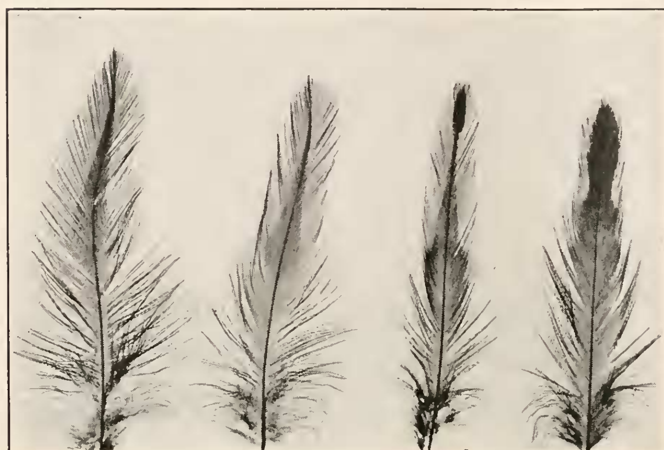
This misconception led us to suspect that the effect of thyroid feeding on the feathering of the male might be indirect, through an augmentation of the so-called luteal tissue, to whose presence in the testes of Campines and Sebright bantams has been imputed the hen-feathered condition of the males of these breeds. No support for this suspicion was obtained from histological preparations, however, and the observation of the thyroid effect in capons completely disposed of the possibility of it. But that the thyroid effect is not independent of the ovary in certain respects is a fact that will be developed in another connection.

The intensity of hen feathering in males of hen-feathered breeds differs with the breed. For instance, the male silver Sebright bantam is an absolute replica of the female, in plumage. The male silver Campine, however, though resembling the female closely, differs from it not only in the possession of sickle feathers but in the structure of the hackles, which are laced in the typical male fashion.

Now the plumage of thyroid-fed Rhode Island Red males, up to the age of 12 weeks, is indistinguishable from that of females. Later on, the males in our experiments developed plumage that is perhaps best described by saying that it was prevailingly male with certain modifications characteristic of the female type.

In place of typical male hackles (Fig. 6, *a*, *b*) with broad marginal lacing of naked barbs, and with sharply pointed tips, thyroid feeding produced hackles with broader and more rounded or truncated tips, and narrower lacing, especially toward the tips, but, more or less irregularly, elsewhere also (*c*, *d*). .Saddle feathers (Fig. 7, *a*, *c*) were similarly affected, the ends being broader and more rounded, and the laced margin furnished with an irregular inner border owing to the development of barbules at points where the barbs are normally naked (Fig. 7, *b*) or in exceptional cases, to the absence of barbules in normal situations. We shall have occasion to discuss these irregularities elsewhere.

The characteristically broad distal lacing of shoulder feathers in normal males (Fig. 8, *a*) was still more strikingly reduced in thyroid-fed males (*b*), often, in fact, completely obliterated (*c*), giving the feathers an undeniably female aspect, as can readily be seen by comparing (*c*) with (*d*), the latter being a feather from the shoulder of a normal female.



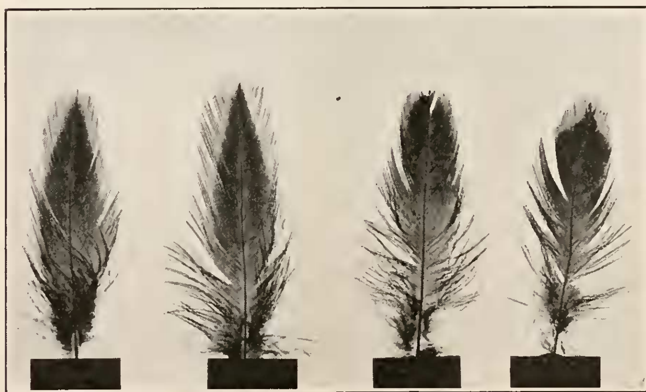
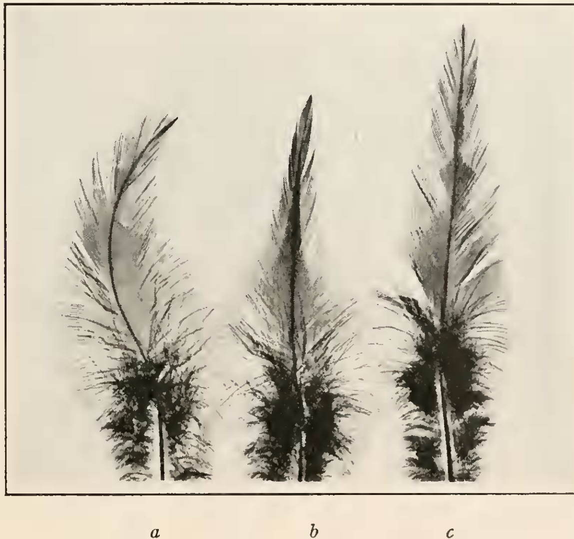




FIG. 6. Hackles from R. I. R. birds about seven months old: *a*, *b*, from a normal male; *c*, *d*, from a thyroid-fed male; *e*, *f*, from a normal female; *g*, *h*, from a thyroid-fed female.

To a less but still striking degree, the hackles of the thyroid-fed males tended to assume the aspect of female hackles. Compare, with this in mind, Fig. 6, *a*, *b*, with Fig. 6, *e*, *f*, *g*, *h*. And note also that, in this series of feathers, the hackles of the female, which are here exceptionally male in structure, are modified in the same direction.

To a still less degree than the hackles do the saddle feathers of thyroid-fed males approximate the saddles of normal females: yet here too the changed shape of the feather and the added barbules and consequent diminished lacing suggest the female type.



*a* *b* *c*

FIG. 7. Saddle feathers from R. I. R. birds about seven months old: *b*, from a normal male; *a*, *c*, from a thyroid-fed male.

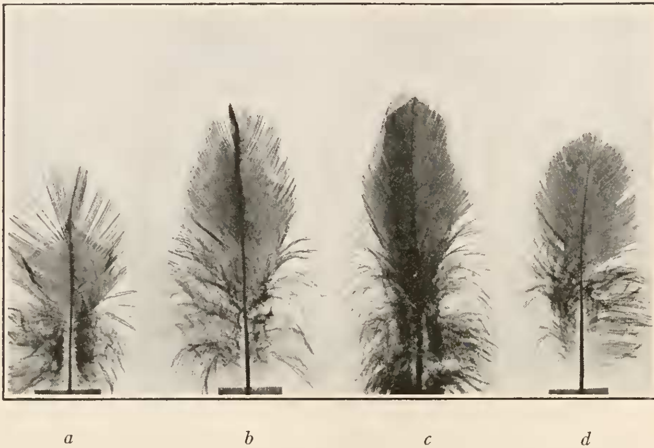
By way of summary it may be said that when male Rhode Island Red chicks were fed desiccated thyroid in daily doses increasing with their weight, two general effects were observed:

1. Their plumage appeared precociously but differentiated later than usual, so that at the age of 12 weeks they were feathered like females. It was to this effect that our earlier observations referred.

2. Their adult plumage, though prevailingly male in type, exhibited characteristics of form and structure, especially in the

hackle, saddle, back and shoulder feathers, that are found ordinarily only in the corresponding feathers of females.[1]

And it may further be said that when the lacing characteristic of the male hackle appears also in the female that the effect of thyroid is to modify it also, as in the male, by the addition of barbules.



*a* *b* *c* *d*

Fig. 8. Shoulder feathers from R. I. R. birds about seven months old: *a*, from a normal male; *b*, *c*, from a thyroid-fed male; *d*, from a normal female.

Thyroid feeding, then, tends to produce hen feathering in the Rhode Island Red male, and, to the extent noted in the last paragraph, in the female also. Toward this result there is no coöperation of the gonad. That the ovary does influence the action of thyroid with reference to certain characters not, however, correlated with sex, will be shown in another paper.

Zoölogical Laboratory,
University of Oregon,
April 7, 1925.

[1] Crew and Huxley (*Veter. Jour.*, LXXIX., No. 10) appear to have seen neither of these effects in their thyroid-fed fowls. The first naturally escaped them, for their birds were too old at the beginning of the experiment to develop it. That the second effect also escaped them may perhaps be attributed to their material and methods. Their 12 birds, of which 6 were males, were $F_1$ hybrids from a cross between a Rhode Island Red ♂ and a Light Sussex ♀, and inherited the black hackles and white ground color from the mother. An amount of thyroid equal to 2 grains per bird was mixed every day into the common ration of wet mash. This dosage was smaller than ours, was not increased as the weight of the birds increased, and was so administered as to leave in doubt just how much each male obtained

# STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES. IV. MALES WITH TWO SETS OF SUPERNUMERARY MALE CHARACTERS.

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In study number three of this series, males of *Cambarus propinquus* and of *Cambarus virilis* which bore supernumerary male characters were described. These supernumerary characters were in the form of copulatory hooks ordinarily found only upon the third walking legs but in these cases present also upon the second or the fourth walking legs. In *C. virilis* the extra hooks were confined to the second walking legs. In *C. propinquus* the additional hooks were found upon either the second or the fourth walking legs. It was predicted that in *C. propinquus*, since either the second or the fourth walking legs might function in carrying the extra hooks, specimens might be found in which copulatory hooks would be present upon both the second and the fourth walking legs.

During the summer of 1924 large collections were made from practically all the streams and some of the lakes of southeastern

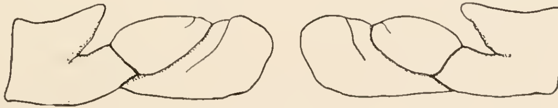

Fig. 1. Diagram illustrating the arrangement of the copulatory hooks upon the third walking legs of a normal male specimen.

Wisconsin and in one stream three males of *C. propinquus* were found which bore supernumerary copulatory hooks upon one or both of the second and fourth walking legs.

## Description of Specimens.

Specimen number one (Fig. 2) is a male of the first form and is 52 mm. long. Normal copulatory hooks are present upon the

third walking legs. Hooks a little smaller than those upon the third walking legs are present upon the right second and the right fourth walking legs. The left second and the left fourth legs possess no hooks.

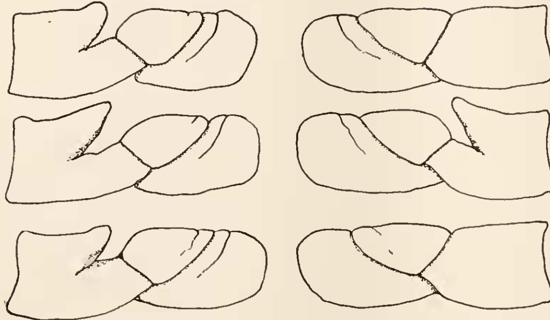

Fig. 2. Diagram illustrating the copulatory hooks upon the second, third and fourth legs as they occur in specimen No. 1.

Specimen number two (Fig. 3), also a first form male, is 43 mm. long and in addition to the usual hooks upon the third legs there are hooks also upon both of the second walking legs and

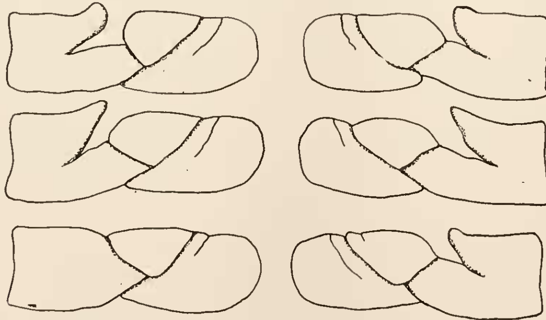

Fig. 3. Diagram illustrating the arrangement of the copulatory hooks upon the second, third and fourth walking legs in specimen No. 2.

upon the left fourth leg. The supernumerary hooks are again distinct but smaller than the normal ones upon the third walking legs.

Specimen number three (Fig. 4) is 47 mm. long and has copulatory hooks upon both of the second, third and fourth walking legs. The hooks upon the second and upon the fourth legs are slightly smaller than the normal hooks.

## Frequency of Occurrence and Distribution.

The three specimens described here were the only ones taken among approximately 3,600 males of *Cambarus propinquus*. The condition may, therefore, be considered rare.

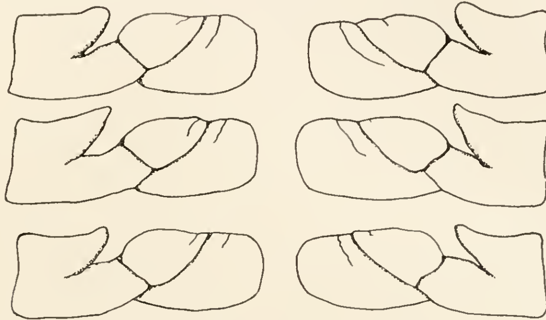

Fig. 4. Diagram illustrating three full pairs of copulatory hooks as they occur upon the second, third and fourth walking legs of specimen No. 3.

All three specimens were taken in a single section of one stream (Pike River near Racine, Wisconsin) and constituted about three per cent. of the males of the entire catch. Thirty-six other streams and rivers examined contained no aberrant specimens of this type.

Male specimens with one pair of supernumerary hooks are sometimes fairly abundant, in some localities representing as high a proportion as seven or eight per cent. of all the males. In Pike River, however, where the three specimens with two supernumerary pairs of hooks were taken, fifteen per cent of the males had one supernumerary pair of hooks, some of them bearing the extra hooks upon the second and others upon the fourth walking legs.

## Discussion.

Some direct and considerable indirect evidence has been accumulated indicating that in female crayfishes with male secondary sexual characters the condition is inherited and should be considered a germinal mutation. It is significant in this connection that all three specimens of the males with two pairs of supernumerary hooks should come from the same locality. It is also suggestive that in this locality there should also occur by far the largest proportion of males with one supernumerary pair of

hooks. If it be assumed that the condition represented by the possession of supernumerary hooks upon the second legs is independent of the condition in which supernumerary hooks occur upon the fourth legs and that both conditions are inherited then it would follow that a combination of the two characters would be most reasonably expected in that locality where both were most abundant. Or, if it be assumed that there is present a cumulative, heritable factor which tends to produce one supernumerary character in the simplex state and added supernumerary characters when doubled then the locality in which doubling would be expected would be that locality in which the highest proportion of single supernumerary characters appeared.

This evidence for the inheritance of the characters described is not convincing in itself but it is important in that it supports the interpretation tentatively advanced for the occurrence of like aberrant structures found in females.

# STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES. V. MALES WITH FEMALE CHARACTERS.

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A number of crayfishes belonging to the genus *Cambarus* in which secondary sexual characters of both sexes were found upon the same individual have been described by Faxon, Hay, Ortman and the writer. All of these specimens with the exception of one described by Hay were females. In some cases there was a full complement of female secondary sexual characters with the addition of one or more male characters and in other cases a female character was lacking while one or more male characters were present. The one exceptional specimen described by Hay was hermaphroditic and possessed both male and female primary and secondary sexual structures.

Male crayfishes in the genus *Cambarus* bearing sexual characters of females have not been noted before and it is the purpose of this paper to describe two specimens which appeared in recent collections made in southeastern Wisconsin. Both specimens were collected from tributaries of the Rock River near Beloit, Wisconsin, the first in July, 1923, and the second in July, 1924.

One is a specimen of *Cambarus virilis*, 65 mm. long and is a second form male normal in every respect except that an oviducal opening exists upon the left third walking leg. An examination of the internal organs shows a normal spermary and normal spermatic ducts. There is no tube of any kind joined to the oviducal opening at the base of the third walking leg. The second specimen is one of *C. propinquus*, 46 mm. long, and is identical in its peculiarities with the first specimen described. It is a normal second form male with all the normal internal and external structures of the male but bearing in addition an oviducal opening at the base of the third walking leg. As in the

previously described specimen there is no duct attached to the aberrant oviducal opening.

## Discussion.

Since all the specimens taken previously which bore secondary sexual characters of both sexes in the same individual have been females or hermaphrodites the impression might easily arise that some mechanism exists which is capable of producing male secondary sexual characters in females but that either the same or some other mechanism prohibits the reciprocal production of female characters in males. Indeed, the following statement with this implication is made by Hay in a discussion upon "Hermaphroditism in Crayfishes."[1] "It would appear, therefore, that in the genus *Cambarus* at least hermaphroditic individuals are females which, owing to some ambiguity of the formative cells in the embryo, have developed to a greater or less degree characters of the opposite sex." The finding of only two males having each a single female character furnishes extremely meager data upon which to generalize but it seems important to demonstrate that crayfishes having secondary sexual characters of both sexes may sometimes be males and that no influence exists in the male which completely inhibits the development of female characters.

[1] Smithsonian Miscellaneous Collections. Sept. 8, 1905.

# PHYSIOLOGICAL STUDIES ON HIBERNATION IN THE POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA* SAY.[1]

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## INTRODUCTION.

An inquiry into the nature and cause of the phenomenon of hibernation has for many years engaged the attention of investigators. The literature on the physiological study of hibernating mammals covers a wide range of research, the most significant of which appears to be into the effects of temperature, food and gaseous exchange. The experimental evidence brought forward by Dubois (10), Pembrey (17), Valentin (25), Weinland and Riehl (29) and others, indicates that hibernating animals placed

[1] A thesis in zoölogy presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

in the presence of an abundance of food and warmth remain impassive. This has led to the conclusion that hibernation is some inherent, deep seated phenomenon. Recent studies by Carlier (8), Rasmussen (18), Sheldon (22) and others, upon the nature and function of the so-called hibernating gland, show it widely present in mammals.

The literature on physiological studies of hibernating insects is not so extensive. Baumberger (1), Bodine (3), Breitenbecher (6), Roubaud (19), Sanderson (20), Tower (24) and others, however, have investigated problems of temperature, moisture, and gaseous exchange as they relate to insects. According to Baumberger (2), hibernation has resulted from the repeated effect of winter upon the species and the rhythmical phenomenon has been determined by the habits of the insect. Roubaud (19) recently advanced the view that a lowered metabolic activity permits a progressive physiological purification. According to this author, two factors, *anhydrobiosis*, and *athermobiosis* (dehydration and absence of heat), in themselves inhibitory, become the unexpected factors of reactivation by favoring the processes of excretion during dormancy.

Most investigators whether dealing with hibernation in vertebrates or invertebrates agree that the influence of temperature in the process is subordinated, and that hibernation is preceded usually by a period of extensive feeding and a consequent reduced metabolic activity. It is also well known that food is an important factor in influencing the habits of animals. The manifold instances of food storage indicate a varying adaptability, the most significant being the storage within the body of the animal of reserve nutrients in the form of fat.

With insects the phenomenon of hibernation is unique, because it may occur in any stage of the life cycle of different insects, from the egg to the adult. It seemed advisable, therefore, to investigate the problem of nutrient storage in insects and to determine the specific mode of its utilization during the progress of hibernation.

This contribution presents the results obtained from physiological studies on the potato beetle, *Leptinotarsa decemlineata* Say, before, during, and after hibernation.

The author desires to express his appreciation to Prof. C. E. McClung, and other members of the Zoölogical Department of the University of Pennsylvania, for generous aid and suggestions. To Doctor J. H. Bodine he is deeply indebted for helpful criticism during the progress of the work and for advising investigation of this problem.

## Material and Methods.

The adult potato beetle, *Leptinotarsa decemlineata* Say, used exclusively in these investigations, was reared from eggs deposited by overwintering adults.

The methods adopted for different lines of investigation varied. The type of cage for determining the effect of various food plants consisted of ordinary six-inch flower pots filled with soil, in the center of which was sunk a 5 x 1 inch glass vial containing water and fresh food plant. The covering to this cage was made of mosquito wire in the form of a circular cage of a diameter to fit snugly within the inner upper rim of the flower pot, and varying in height from 12 to 18 inches. These cages were set in troughs of soil in an outdoor breeding house. A more elaborate cage consisted of a wooden framework (20 x 18 x 14 inches), constructed of inch pine, enclosed with mosquito wire and set on

Fig. 1. Types of cages used in the food experiments. This figure shows two types: (1) circular cages composed of flower pots sunk in the soil with mosquito wire tops; (2) large oblong cages constructed of inch pine, covered with mosquito wire and set on the surface of the soil. Foliage was kept fresh in the cages by inserting them in vials of water.

the surface of the soil in the breeding house or in the field (Fig. 1). In all cages it was impossible for beetles to escape or for others to find entrance from the exterior. Access for the worker was by a convenient opening on the top or side of the cage.

The water content was obtained by first weighing and slitting the animals and afterwards placing them in an oven at a temperature between 90–95° C., until a constant weight was obtained. The percentage of water was computed on the basis of dry weight.

Although the extraction of fat from the body of an organism with solvents cannot be achieved quantitatively without change in the nature of the compounds in which the fatty acids are present, this was not considered an objection in these experiments as the result desired was the total fat content in whatever form it might occur. The tissues, however, had to be dried and powdered. To prevent partial oxidation of the more unstable unsaturated acids, the drying took place at room temperature, or in an oven at a temperature not exceeding 30° C. The extracting solvent used extensively was a mixture of equal proportions of ether and alcohol, which was warmed and renewed several times by decantation. In addition, the method described by Voltz (28), and modified to suit the material under investigation was also used. Calculations were made in percentage based on dry weight of the organism.

Metabolism determinations (respiratory exchange) were obtained by methods essentially similar to those described by the author (12) in a previous publication. Other methods used will be described in the text under appropriate headings.

## Experiments and Discussion.

### *Food Experiments.*

The effect of different food plants on potato beetles of the first generation emerging from pupation was studied. Altogether, during June, 1923, and 1924, about 800 beetles were divided into four main groups and fed on various food plants as follows:

Group 1. The foliage of potato, tomato, eggplant and peppers.
Group 2. The tubers or fruit of the above plants.
Group 3. The foliage of potato for six days, followed by sliced

non-solanaceous foods like beets, carrots, squash and cucumbers.

Group 4. Non-solanaceous foods exclusively.

*Number of Days Feeding Before*[1] *Hibernation.*

The number of days feeding on different food plants before hibernation took place is shown in Tables I.–IV., and in Fig. 2. Group 1, fed on foliage required an average of 16 days, whereas group 2, fed on tubers and fruit required an average of 13 days. Evidently, to accumulate sufficient nutrients necessitated a more extensive feeding on foliage than on tuber or fruit. The experiment with group 3, fed on potato foliage for six days followed by a period of feeding on sliced non-solanaceous foods, resulted in, first, an increase in the number of feeding days essential for nutrient storage to an average of 23.6 days; second, a mortality of 23 per cent.; third, unsuccessful hibernation of 32 per cent. (which died during the process).

TABLE I.

NUMBER OF DAYS FOR BEETLES FED ON FOLIAGE TO ENTER HIBERNATION.

| Potato. | | Tomato. | | Eggplant. | |
|---|---|---|---|---|---|
| Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. |
| 75.......... | 14 | 100.......... | 15 | 80.......... | 11 |
| 25.......... | 21 | | | 20.......... | 20 |
| Average..... | 17.5 | | 15 | | 15.5 |
| *Cages Receiving No Water.* | | | | | |
| 80.......... | 11 | 10.......... | 11 | 90.......... | 18 |
| 10.......... | 13 | 80.......... | 13 | 10.......... | 20 |
| 10.......... | Died | 10.......... | Died | | |
| Average..... | 12 | | 12 | | 19 |

The general effect with group 4, fed on sliced non-solanaceous foods exclusively and those members of group 1, which were fed on pepper plants or its fruit, was one of starvation. Some died after 7 days; others lived for 41 days but were unable to hiber-

[1] Potato beetles hibernating in July or November exhibit the same physiological reactions.

## Table II.

Number of Days for Beetles Fed on Tubers and Fruit to Enter Hibernation.

| Potato. | | Tomato. | | Eggplant. | |
|---|---|---|---|---|---|
| Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. |
| 80 | 13 | 10 | 11 | 50 | 11 |
| 20 | 15 | 80 | 13 | 50 | 18 |
| | | 10 | Died | | |
| Average | 14 | | 12 | | 14.5 |
| *Cages Receiving No Water.* | | | | | |
| 80 | 11 | 10 | 11 | 10 | 11 |
| 20 | 13 | 90 | 18 | 90 | 13 |
| Average | 12 | | 14.5 | | 12 |

## Table III.

Number of Days for Beetles Fed on Foliage Followed by Sliced Tubers and Non-solanaceous Foods to Enter a State of Hibernation.

| Potato. | | Squash. | | Cucumbers. | | Beets. | | Carrots. | |
|---|---|---|---|---|---|---|---|---|---|
| Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. |
| 75 | 15 | 80 | 25 | 40 | 15 | 40 | 13 | 40 | 15 |
| 25 | 25 | 20 | 35 | 50 | 21 | 50 | 38 | 10 | 38 |
| | | | | 10 | Died | 10 | Died | 50 | Died |
| Average | 20 | | 30 | | 18 | | 25.5 | | 25 |

## Table IV.

Beetles Fed on Non-solanaceous Foods Showing Number of Days before Death.

| Squash. | | Cucumbers. | | Beets. | | Carrots. | | No Food. | |
|---|---|---|---|---|---|---|---|---|---|
| Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. | Per Cent. Beetles. | Days. |
| 40 | 37 | 40 | 15 | 60 | 37 | 40 | 15 | 100 | 11 |
| 60 | 45 | 40 | 26 | 40 | 39 | 60 | 38 | | |
| | | 20 | 34 | | | | | | |
| Average | 41 | | 25 | | 38 | | 24 | | |

nate. In control cages where nothing was fed, the beetles died after 11 days.

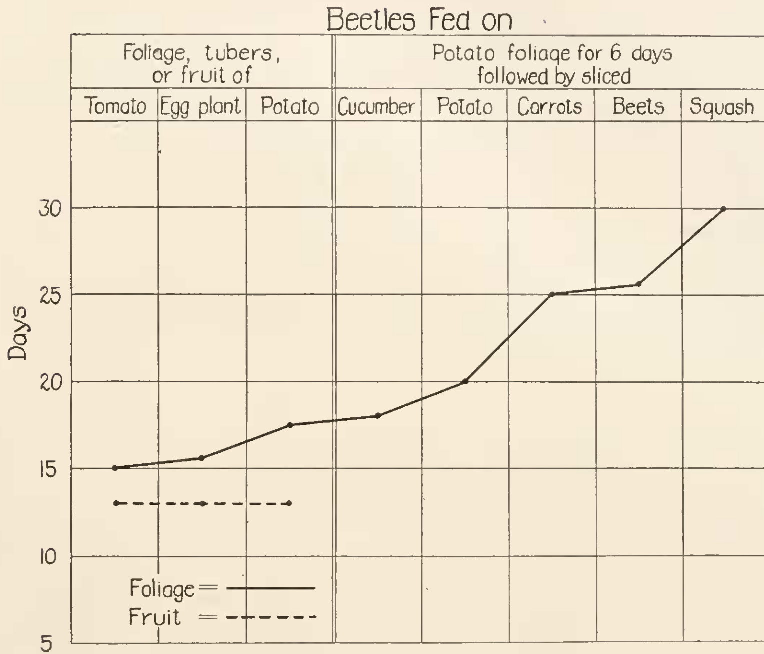


FIG. 2. Represents the number of feeding days required on different foods before the beetles entered a state of hibernation. Days are represented as ordinates, different foods as abscissa. The solid line curve shows the average number of days feeding on different foods before hibernation. The dotted line shows the average number of days feeding on tuber or fruit before hibernation.

In duplicate series of experiments conducted at the same time, certain cages received water to replace usual evaporation from the soil, others received no water. The addition of water to cages containing normal food plants decreased the feeding period from an average of 14.5 to 13.4 days. In control cages receiving only water the beetles died after 16 days. In cages containing non-solanaceous foods, the addition of water seemed to prolong the life of the beetles without inducing hibernation.

*Breeding Activities.*

It is perhaps essential to emphasize that potato beetles of the first generation used exclusively in these investigations indicated

no breeding activities before dormancy set in. The beetles hibernated during July and August and remained in that state until the following season. Other experiments conducted by confining first generation beetles with normal food plants in field cages, produced similar results. During fall and winter these hibernating beetles were frequently removed from the soil in the cages, and sections made of the testes revealed mature sperm in abundance. Dissections of the females yielded immature ova. These results are contrary to those obtained by Tower (24), who states, "The first brood on emergence feeds for a few days and then deposits eggs for a second generation. The second generation does not develop the germ cells nor show any reproductive activity until after it has passed through a period of hibernation or æstivation."

### *Metabolism During Feeding Experiments.*

During the progress of feeding a veritable storing of food in the form of fat takes place in the adipose tissues of the animal (Fig. 4). It has been pointed out above that a numerical difference in days exists, for nutrient storage when different foods are fed. From metabolism determinations it seems possible to correlate the results procured during the feeding experiments with the oxygen consumption. In Fig. 3, the oxygen intake per gram organism in cubic millimeters per hour is shown. From an examination of this figure it is evident that a reduced oxygen consumption takes place when tuber or fruit material, as compared to foliage, is fed. Obviously then the process of converting tuber material required less oxygen. The analysis of food plants, given in Table V., shows the amounts of carbohydrate and fat

Table V.

Showing Analysis of Food Plants in Percentage.

| Food Plant. | Water. | Protein. | Fat. | Carbohydrate. |
|---|---|---|---|---|
| Beets | 87.5 | 1.2 | 0.1 | 9.4 |
| Carrots | 88.2 | 0.7 | 0.4 | 8.9 |
| Cucumbers | 95.4 | 0.6 | 0.2 | 3.0 |
| Eggplant | 92.9 | 0.9 | 0.3 | 4.9 |
| Potato | 78.3 | 1.7 | 0.1 | 17.7 |
| Squash | 88.3 | 1.1 | 0.5 | 8.6 |
| Tomato | 94.3 | 0.7 | 0.4 | 3.8 |

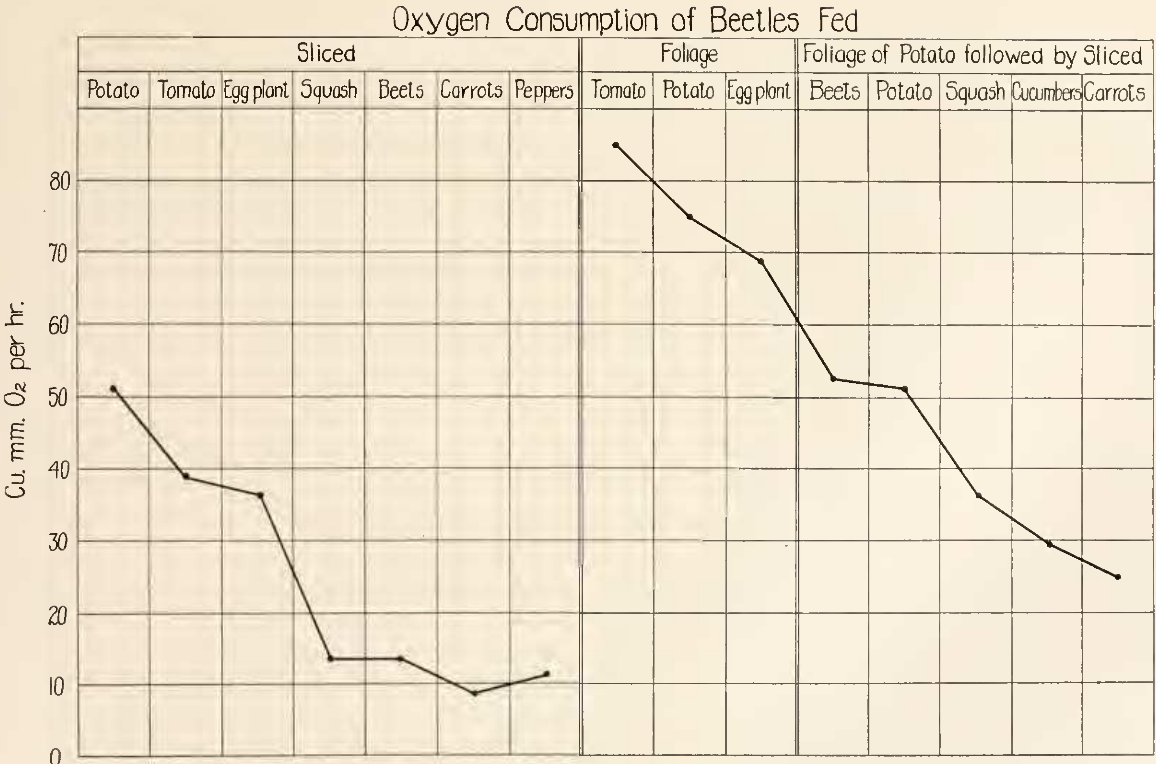


FIG. 3. Ordinates shows the oxygen intake in cubic millimeters per hour per gram body weight of beetles fed on different foods represented as abscissas. By comparison it is noted that distinctive differences in oxygen consumed are evident when potato beetles are fed tubers and fruit as compared to foliage of the same plants. This figure also shows the low oxygen intake when fed non-solanaceous foods.

present in non-solanaceous foods as compared to potato tuber and foliage. It is known that a diet rich in carbohydrates, as for example, turnips or potatoes, increases the proportion of lower fatty acids, while a diet rich in proteins diminishes this proportion. If the percentage of the lower fatty acids increases with the amount and kind of food, this fact may explain the results shown in the above experiments, since tuber material is rich in carbohydrates.

It was previously pointed out that members of group 4, fed non-solanaceous food plants and those beetles fed on peppers, in general, gradually starved. The respiratory metabolism of such animals manifests an extremely reduced oxygen consumption and (in other experiments to be discussed later) a decreased $CO_2$ output. In control experiments, where nothing was fed, the oxygen intake similarly was exceedingly diminished.

## Preparation for Hibernation.

Of significance are the variations in weight recorded during the preparation for hibernation. First generation beetles as they emerged from pupation and before feeding averaged 0.1260 grams in weight. After feeding on foliage of the potato plant for ten days, the weight averaged 0.2627 grams. A state of quiescence or *prehibernation* [2] followed, varying from three to five days and was accompanied by a reduction of the water content and elimination of the waste material from the digestive tract, giving an average weight of 0.1879 grams. The increase in weight accounts, in part, for the accumulation of reserve nutrient material in the adipose tissues, and may, in addition, indicate growth of somatic and germinal cells. Furthermore, during *prehibernation* a gradual lowering of all vital activities takes place, the beetles afterwards entering the ground to hibernate.

Determinations of the water and fat content were made upon groups of beetles as shown in Fig. 4. From an examination of these it is evident that during active feeding, the average water and fat content was 76.4 and 7.8 per cent. respectively. During *prehibernation*, the average water and fat content was 59 and

[2] The term *prehibernation* is used to designate the quiescent state that occurs when feeding ceases and before the beetles burrow in the ground.

20 per cent. respectively. When the beetles burrowed in the ground the water content averaged 56 per cent. and the fat content 29 per cent. The results of these experiments indicate quite clearly that a loss of 20 per cent. in the water content and a gain of slightly over 20 per cent. in the fat content took place. Tower (24) obtained with this species a reduction of 27 per cent. in the water content. With hibernating grasshoppers Bodine (4) found a reduction of 13 per cent.

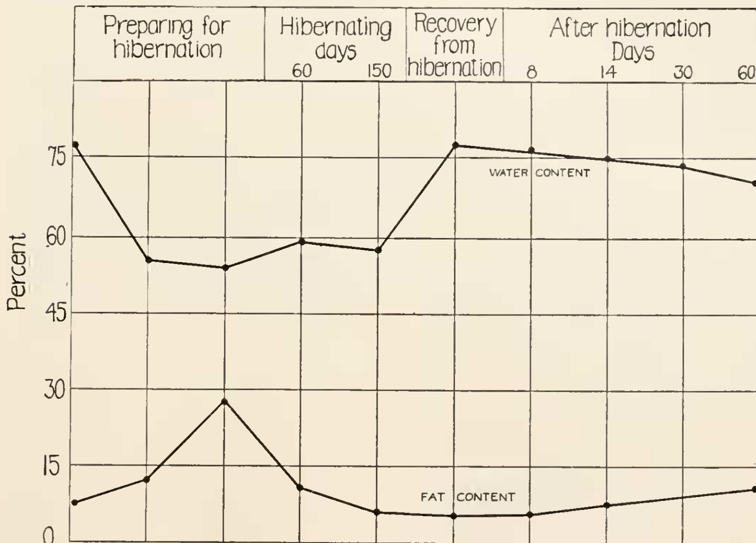


FIG. 4. Ordinates show the percentage of water and fat content of potato beetles, before, during and after hibernation. Note the reduction in the water content and increase in fat content as the beetles enter hibernation, and the extensive depletion in fat during the first few months as compared to the remaining period of hibernation.

The view commonly held is that a diminished water content makes protoplasm able to withstand extremes of temperatures. With potato beetles this does not appear to be valid, since invariably they must burrow in the ground to a considerable depth (10–18 inches) presumably to successfully escape extremes of temperatures. Experimentally the influence of greater variations in temperatures was determined by permitting only several inches of soil to intervene between the dormant beetles and the outside air. The results of such treatment upon hibernating

beetles during the winter, produced a mortality of 100 per cent. In this species a reduction of the water content probably does not proceed to a sufficient degree to prevent injury by freezing temperatures, hence necessitating their further protection by burrowing in the ground.

During the progress of hibernation, continued investigations have shown that the water content remains practically constant, although with a general diminution of fat, a slight increase in the water content may follow. Extremely significant is the excessive depletion in fat that obviously takes place during the first two months (51 per cent.) as compared to the remaining five months (49 per cent.) of hibernation.

In this connection it is of interest to cite, for comparison, the results of other investigators concerning fat consumption during hibernation. Victoroff (26) with frogs found a consumption of 23 per cent. Investigators with mammals demonstrated the prevalence of a large fat content which gradually disappears during the period of torpidity. Thus Voit (27) found that in the marmot the adipose tissue was more than 30 per cent. of the weight of the body. Valentin (25) found the adipose tissue in the marmot contributed about twenty times as much food as the hibernating gland. He also observed that the depletion in fat which occurred during the first few months was greater than after five months hibernation. Similarly Carlier (8) found that almost one half of the fat disappeared in the hedgehog during the first few months of dormancy. It is of interest to discover this general conformity between a mammal and an insect in the utilization of nutrient material during the course of hibernation.

*Hibernating Gland.*

Many investigators mention a hibernating gland which is said to be commonly found in mammals (hedgehog, marmot, bat, shrew, rat, mole, beaver, squirrel, weasel, martin, badger, rabbit, guinea pig, cat, dog and even in man). Rasmussen (18) who has reviewed the literature on this subject extensively, is of the opinion that it is distinct from adipose tissue. Sheldon (22) recently concluded that "the so-called hibernating gland is essentially a form of adipose tissue which retains its embryonic characteristics for a more or less indefinite length of time, . . .

it intergrades with ordinary adipose tissue and under favorable conditions is transformed into it."

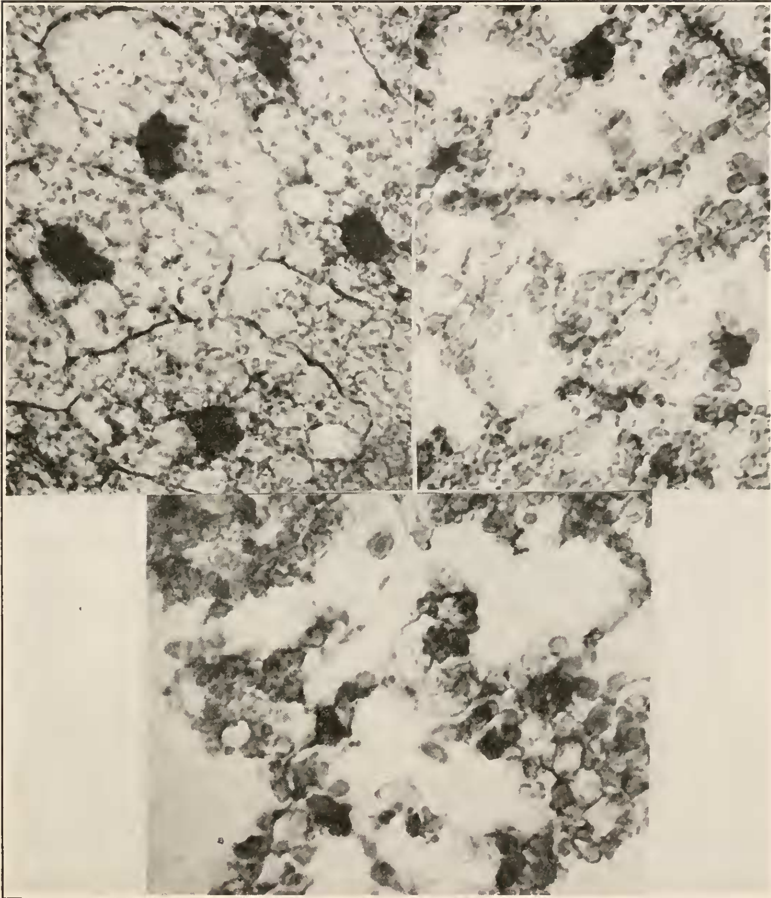

FIG. 5. Upper left. Section of the fat body or adipose tissue of potato beetle before hibernation. Note the numerous fat vacuoles partly masking the large nucleus located centrally within the cell, and the minute granules dispersed among the vacuoles. Flemming fixation, stained with hematoxylin. Photographed with achromat 2 mm., lens, 1.3 numerical aperture with 12 × compensating eye piece.

FIG. 6. Upper right. Section of the fat body after one month of hibernation, showing the decreased size of the fat vacuoles and nucleus, the large and prominent albuminoid granules grouped along the periphery of the cell exhibiting open spaces. Staining and enlargement as in Fig. 5.

FIG. 7. Section of the fat body after five months hibernation, showing the almost complete absence of fat vacuoles. The prominently large albuminoid granules are grouped in masses, the absence of cell outline of the fat body and the almost entire disintegration of the nucleus. Staining and enlargement as in Fig. 5.

Whether it is ordinary adipose tissue or a special gland that functions in the mammal as a storage for nutrient material, with the potato beetle, and perhaps with insects in general, it seems quite certain that adipose tissue serves that purpose. To determine some of the morphological changes that take place during hibernation sections were made of the adipose tissues of the potato beetle. During *prehibernation* (Fig. 5) the sections show it to be composed of regular cells, oval in general outline and filled with conspicuous fat vacuoles of more or less uniform shape and size which extensively overlap and partly mask the actual contour of the large nucleus. Barely distinguishable are diminutive albuminoid granules dispersed among the vacuoles. The sections of beetles in hibernation for one month (Fig. 6) are strikingly different in appearance from those of non-hibernating ones. In the former we find that the fat vacuoles have become much smaller and that the nucleus has undergone disintegration and diminution in size. The most striking objects are the albuminoid granules aggregated along the periphery of the cells of the fat body, or clustered around the nucleus. The sections of adipose tissues of animals in hibernation for the longest period (six months) show clearly the prominently large albuminoid granules clustered in groups, exhibiting many open spaces (Fig. 7). Indications of a dissolution of the cells of the fat bodies and a dispersal of the albuminoid granules are quite evident.

The author compared Figs. 5–7, with those published by Rasmussen and Sheldon of sections taken from the hibernating gland in mammals, and the similarity is indeed very striking.

The existence of albuminoid granules or uric acid concretions indicates an active metabolism in the fat body. According to Lang (15) the fat body of larvae of insects is rich in fat and poor in concretions of uric acid before metamorphosis, while in the adult the opposite is true. Fabre (11) thinks that the adipose tissue serves the purpose of a urinary organ, since urates are formed within the cells. Both Graber (13) and Landois (14) regard it as a single many lobed lung (owing to the many fine branched tracheal endings in the fat body). Similarly Roubaud's view of the necessity of a physiological purification during

hibernation, based upon observing adipose tissue loaded with urate crystals before hibernation, must be regarded as signifying that it serves the purpose of an excretory organ.

There appears no valid reason for assigning to the fat body a urinary function. In the hibernating animal the metabolic activity of most cells is reduced to a minimum, and in the fat cells, judging from the respiratory quotient, it is most active. Hence the cells become charged with an accumulation of albuminoid granules and other products. The urates found in these cells are not an indication of their special urinary function, but of a more active metabolism at a period which serves the animal best. This may occur before or during metamorphosis, or throughout the progress of hibernation.

## The Metabolism of Active, Starving and Hibernating Beetles.

A knowledge of the respiratory metabolism of active, starving and hibernating beetles seemed desirable for comparing the chemical changes involved. Of singular interest are the analogous rates of $CO_2$ output procured from beetles emerging from hibernation and pupation (Figs. 8–9), indicating, perhaps, a condition of physiological youngness or purification (Roubaud) as a result of hibernation. Bodine (4) found hibernating grasshoppers produced a higher rate of $CO_2$ output than growing animals kept at the same temperature, suggesting he states, "that animals remain young throughout the period of hibernation." The $CO_2$ output of potato beetles throughout the progress of hibernation invariably indicates a reduced metabolic activity, in some respects comparable with that of starving animals (Fig. 9). Indeed, there appears to be a parallelism in respiratory metabolism between starving and hibernating forms. In the former, however, the velocity of reaction of the life processes continues to function most actively and reserve substances are rapidly depleted, a condition eventually leading to the death of the animal. In the latter most of the life activities are considerably depressed and nutrient material is, therefore, used sparingly.

With older beetles, the metabolic activities are also extremely reduced, but not to the extent met with in hibernating or starving

FIG. 8. Ordinates represent the $CO_2$ output per hour per gram body weight of organisms. Note the reduced $CO_2$ output of hibernating and starving as compared to active animals, indicating a parallelism in metabolic activity between starving and hibernating beetles.

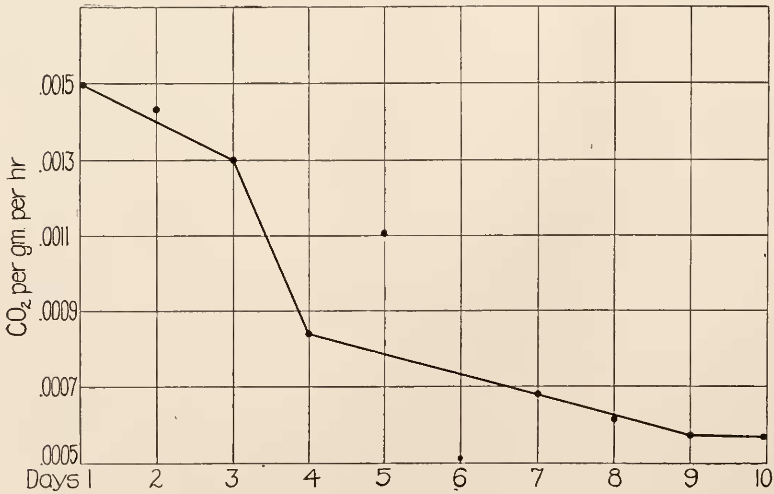


FIG. 9. Ordinates show the $CO_2$ output per hour per gram body weight of beetles during the progress of starvation until death. Abscissas represent days.

forms. The overfed and old state of the cell, as suggested by Baumberger (1) has perhaps reduced permeability to a wide degree. In old age or pathological conditions, according to Mathews (16), an accumulation of inactive ingredients or of acid in the cells, retards metabolic activity. In a measure, we are thus enabled to interpret the specific differences in metabolism that may take place under varying conditions in the organism. Especially noteworthy is the utilization of reserve nutrient material in the hibernating animal discussed further on.

### *Respiratory Quotient.*

It is well known, experimentally, that when substances catabolized are chiefly fat, a reduced respiratory quotient results, whereas the catabolizing of carbohydrates and protein produces a higher quotient. According to the investigations of Dubois (10), Pembrey (17), Weinland and Riehl (29), Valentin (25) and others, reduced respiratory gas exchanges in the marmot occur during hibernation with strikingly low respiratory quotients (0.44–0.72). The views advanced to explain a quotient not consistent with the utilization of fat are many. Certain investigators (Dubois, Pembrey, Valentin) perceived that although mammals consumed no food during dormancy an increase in weight very often took place. It was, therefore, assumed that the oxygen consumed, greater than is necessary for fat oxidation, was in some way retained in the animal. Dubois also discusses building of acetone which he found accumulated in the blood-urine of hibernating mammals. It is known that of the normal fatty acids from butyric to decoic acid, only those with an even number of carbon atoms give rise to a marked increase in acetone formation. Dakin (9) and others have shown that acetone is derived from the decomposition of acetoacetic acid. This supports Knoop's theory of the B-oxidation in which two or some multiple of two carbon atoms are lost during oxidation, and offers a possible explanation that intermediate stages supervene throughout the process of the utilization of fat. Furthermore, Weinland and Riehl claim that during awakening from dormancy the marmot needs carbohydrates, and that the required carbohydrates come partly from substances produced

in the body during hibernation. But it is not certain whether they are produced from fat, protein, or both. Voit (27) holds that fat may form sugar, which in turn can be stored up as glycogen.

The respiratory quotients obtained with the potato beetle demonstrate a wide degree of variation during different periods of hibernation. The respiratory quotient is lowest during the

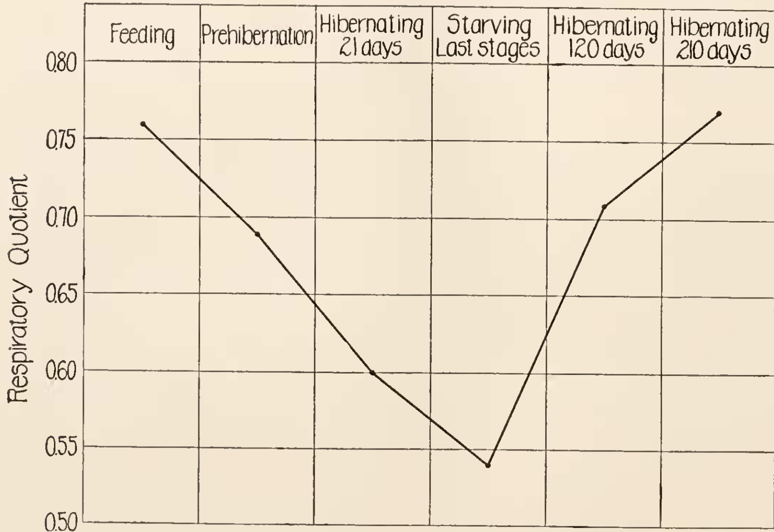


FIG. 10. Ordinates show the respiratory quotient based on respiratory metabolism ($CO_2$ output and oxygen intake) of active beetles and of those after varying periods of hibernation as compared to starving animals.

first few months of dormancy and increases progressively. A noteworthy increase in the quotients results when the animals awaken to activity (Fig. 10). This may agree with the theory brought forward by Weinland and Riehl, that carbohydrate is being oxidized for the purpose of awakening. On the other hand, during acute stages of starvation, potato beetles evince an exceedingly reduced respiratory quotient (0.54). Dubois has shown that a hibernating mammal loses as much in weight in 160 days as a starving animal in 12 days. Since it is recognized that reserve nutrient materials are rapidly depleted during starvation it is possible, perhaps, to correlate the diminished respiratory quotient noted during the early phases of hibernation

with the immense decrease in the fat content which likewise results at this period.

## Recovery from Hibernation.

In the preparation for hibernation the potato beetles pass through a quiescent phase which has been designated as *pre-hibernation.* Likewise on emergence from dormancy there is a period of quiescence known as recovery. From results obtained on respiratory metabolism during recovery and before feeding, it is evident that the increase in $CO_2$ output is very gradual, lasting for six days (Fig. 11). The examples upon which the

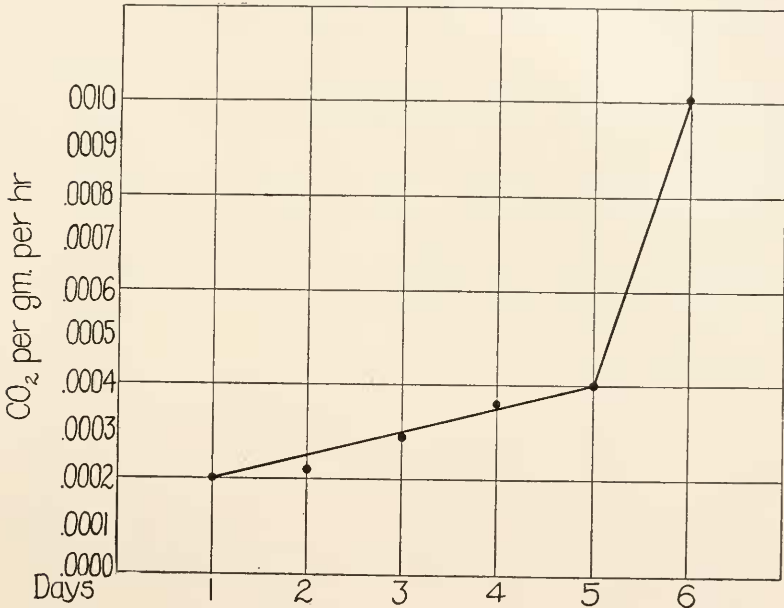


Fig. 11. Ordinates show the average $CO_2$ output per hour per gram body weight of beetles during recovery from hibernation. This figure is based on group of beetles hibernating for six months.

above figure is based, comprised a group of beetles in hibernation for six months. After varying periods of dormancy, if potato beetles are removed from the ground and placed with food, we find they require from six to thirty days for recovery before feeding. Those that have been in hibernation for over two months require 30 days, those for three months 12 days, and

those five or more months 6 days. During the interval of restoration they are decidedly inactive, negatively heliotropic and do not feed.

Extensive experiments carried out during the winter with the view of hastening recovery proved fruitless. In every instance hibernating beetles when removed from the ground and placed with food persistently indicated tendencies to burrow themselves in again. In those cages not provided with soil the beetles remained inactive on the side or bottom of the cage without feeding.

Both Bodine (4) and Sanderson (20) found that the rate of recovery was quickest with animals brought in later in the hibernating period. Sanderson, however, concluded that subjecting hibernating forms to low temperature had the effect of producing a more complete rest than at a higher temperature. In view of additional experiments performed by the author a different interpretation may be attributed to the above results. For example, beetles reared in the greenhouse hibernated the latter part of March and were kept at a comparatively high temperature (65–95° F.) in the greenhouse. On July 11, they were found issuing from the ground in the cages, exhibiting normal activities such as feeding, mating, etc. In these experiments, therefore, cold was not essential for a complete rest. Furthermore, the depth at which potato beetles normally hibernate in the soil (10–18 inches) precludes the assumption of a specific influence of low temperature, or of the necessity of subjecting them to prolonged periods of cold. On the contrary, experimental evidence demonstrates a rhythmical period of rest irrespective of temperature.

*Adjustment of the Water Content.*

Of paramount importance to the animal during recovery and before feeding is possible, is an adjustment of its water content to normal. The need for actual contact of the animal with water at different intervals seems to be essential. Whether moisture may also be absorbed through the integument is not definitely known.

In the following experiments beetles recovering from hibernation were kept in cages with abundance of food under the

same temperature conditions in the greenhouse. One lot frequently received water, the other lot was not watered. The addition of water (a drenching of the cage) had an activating effect, since the beetles eventually became active and fed normally on the plants. In those cages not receiving water the beetles were apparently unable to recover and finally perished. The lack of actual contact of the animal with water evidently hindered recovery.

Dissected hibernating beetles invariably revealed accumulated waste products in the rectum. In normal animals its expulsion is necessary before activity and feeding is possible. It seems reasonable that water actually is imbibed by the animal during recovery to aid in the elimination of inert substances from the digestive tract.

## Catalase and Oxidase Activity.

It seemed desirable to determine if other factors concomitant with a lowered metabolic activity are involved in the hibernating animal. There are many facts in the literature tending to show that the power of decomposing hydrogen peroxide and the power to blue guaiacum by the aid of peroxide are the specific property of certain substances. Since the recognition of catalase as a specific enzyme, its occurrence and distribution in various animal and vegetable tissues has been investigated by a number of observers.

Certain investigators are of the opinion that the accumulation of hydrogen peroxide would undoubtedly prove harmful to the organism, and that the function of the catalase is to destroy the hydrogen peroxide as fast as it is formed. Others hold that the function of catalase may be to prevent the excessive oxidation of organic substances in the living cell. Schoenbein (21), however, has shown that substances which can bring about the decomposition of hydrogen peroxide catalytically, can also greatly increase its oxidizing power, and in proportion as a substance is able to decompose the peroxide so also it can accelerate oxidation. The power to decompose hydrogen peroxide is held by Spitzer (23) to be a measure of the oxidizing power of various animal tissues. Dakin (9), however, is of the opinion

that there is no trace of evidence to prove that catalase is directly concerned with oxidation, since catalase, he states, "only liberates inactive molecular oxygen when decomposing hydrogen peroxide." Burge (7) found a quantitative relation between the amount of catalase and oxidation; an increase of oxidation in young and a decrease in old animals. Similarly Bodine (5) with certain insects obtained a decreased catalase content with increasing age and with animals subjected to starvation.

The author made determinations of the activity and total content of catalase in hibernating, starving, young and old potato beetles, using for this purpose the same methods described by Burge. The results of these experiments are graphically represented in Figs. 12–13. With hibernating animals the total catalase content per gram body weight is lower than with either

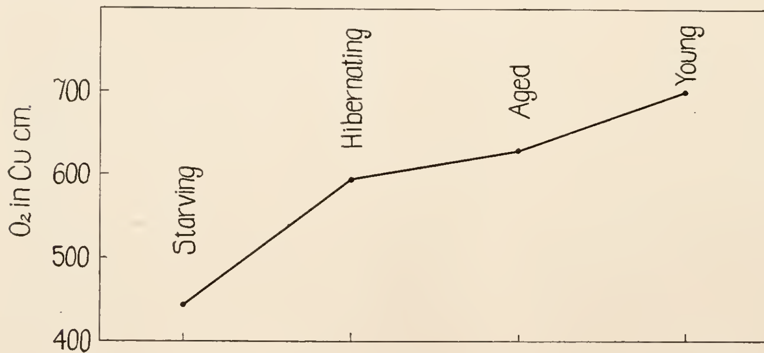


FIG. 12. The average total catalase content per gram body weight of different groups of beetles as measured by the oxygen evolved. Ordinates show the oxygen evolved in cubic centimeters.

young or old beetles; starving animals indicate the lowest catalase content. In Fig. 13, the catalase activity per gram body weight per minute is recorded and strikingly indicates a seeming parallelism in catalase activity between hibernating and starving animals. Moreover, the extremely reduced catalase activity indicates a correlation with a diminished respiratory metabolism in hibernating and starving animals.

Experiments to determine the oxidase activity were made by using guaiac, P-diamino benzene with peroxide upon tissues, organs and body fluids. Upon hibernating animals the reaction

were negative since no typical characteristic blueing of guaiacum took place. Similar tests performed with active and starving beetles, gave striking reactions (blueing of guaiacum) with esophagus, stomach, tip of rectum, tissues, body fluids, testes and immature ova.

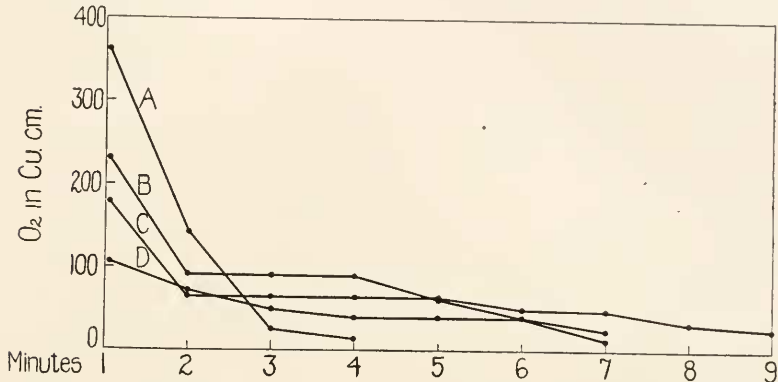


FIG. 13. The catalase activity per minute per gram body weight of organism. Ordinates show the oxygen evolved in cubic centimeters, abscissas as minutes. *A*, young beetles; *B*, aged beetles; *C*, hibernating beetles; *D*, starving beetles.

Although the catalases and oxidases may possess specific functions in the active animal, in the hibernating forms these enzymic activities obviously become greatly reduced or seemingly absent.

## SUMMARY.

1. The results of the food experiments indicate that potato beetles of the first generation when fed on tubers and fruit of the potato, tomato, and eggplant require 13 days feeding to enter hibernation, while those fed on foliage of the same plants need 16 days. Beetles fed on potato foliage for six days followed by a non-solanaceous food required 23.6 days feeding before hibernation, and produced a mortality of 55 per cent. Those fed on pepper plants or on non-solanaceous foods do not hibernate, but die of starvation.

2. Respiratory metabolism determinations show that the oxygen consumption of beetles fed on tubers or fruit of solanaceous plants is less than of those fed on foliage. When fed on non-solanaceous foods, the reduced oxygen intake recorded was

comparable with the oxygen consumption of starving animals. A reduced $CO_2$ output occurs throughout the progress of hibernation.

3. Preparation for hibernation follows a period of extensive feeding and consists in an accumulation in the adipose tissues of 29 per cent. fat. During prehibernation a reduction of the water content of 20 per cent., the elimination of waste products from the digestive tract and a lowered metabolic activity of the animal take place.

4. The greatest depletion in fat occurs during the first few months of hibernation. In the metabolism of the fat body, the fat is replaced by albuminoid granules and other products. Sections of adipose tissue before and after hibernation are comparable to sections of the hibernating gland of mammals.

5. The respiratory quotient is lowest during the first few months of hibernation and highest at the termination of dormancy (0.60–0.76). In hibernating animals possessing insufficient nutriment and in starving animals, the respiratory quotient is exceedingly low (0.54). There appears to be a parallelism between a rapid depletion of reserve food and a low quotient.

6. Recovery depends upon the length of time potato beetles spend in hibernation. It is more rapid with those in hibernation for the longest time. Actual contact of the animal with water seems necessary to restore its water content before feeding is possible.

7. Catalase activity of hibernating beetles is greatly reduced and their total catalase content is lower than that for old or young active beetles. There appears to be a correlation between the reduced catalase activity and diminished respiratory metabolism in hibernating and starving animals.

Although active beetles give very striking oxidase reactions, no characteristic blueing of guaiacum was obtained with any organ, tissue, or body fluid of hibernating forms.

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Vol. XLIX December, 1925 No. 6

BIOLOGICAL BULLETIN

# ON THE FEEDING REACTIONS AND DIGESTION IN THE CORAL POLYP *ASTRANGIA DANÆ*, WITH NOTES ON ITS SYMBIOSIS WITH ZOÖXANTHELLÆ.

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In comparison with the extensive literature on the feeding reactions and the digestion in actinians, these phenomena in madreporarian polyps have been studied by a few authors only. Our knowledge of these phenomena is chiefly due to Carlgren (1905, *Caryophyllia*), Duerden (1906, *Fungia* and *Favia*), Carpenter (1910, *Isophyllia*), and Vaughan (1912, various West Indian reef-corals). In the following pages the results are given of an investigation on the feeding reactions and the digestion in the coral-polyp *Astrangia danæ* Ag.

The work on which this paper is based was made possible by a subvention of the Netherland-America Foundation, which enabled me to visit the United States for some months. It was carried on in the Marine Biological Laboratory at Woods Hole (Mass.) in August and September, 1924. I want to thank here the Director of this Laboratory, Dr. F. R. Lillie, for the opportunity I obtained to work some time at this institution.

The study of the digestion in *Astrangia* is meant as a base for the further study on the digestion in the polyps of reef-corals. As is well known, the polyps of reef-corals contain a large number of unicellular algæ, the zoöxanthellæ, which are located in the entoderm-cells. Although this association of coral-polyps with algæ is generally considered as a kind of symbiosis, there are very few statements on the advantages which both organisms derive from it. One of the ways to investigate this problem is the comparison of the physiology of a form with symbiotic algæ with

that of a form without these organisms. Now *Astrangia danæ* generally is completely devoid of zoöxanthellæ, but during the time I worked in Woods Hole I obtained some colonies which were strongly infected with these algæ. The fact that my material consisted partly of polyps without zoöxanthellæ and partly of those infected with the symbiotic algæ enabled me to make a comparison of the feeding and digestion in these different kinds of polyps. As I disposed during the first half of my time in the Marine Biological Laboratory only of polyps without zoöxanthellæ, the larger part of my experiments were made with such uninfected polyps. Afterwards, when comparing the phenomena of digestion in polyps without zoöxanthellæ with those found in uninfected polyps, I could at least partly investigate the rôle of the zoöxanthellæ in the feeding of the polyps which are infected by these algæ.

I kept the polyps of *Astrangia* easily alive in glass-vessels with running sea-water, and at the end of a month they were in the same healthy state as at the beginning of the experiments. Some colonies even had enlarged in size by budding. The fact that *Astrangia* may be kept alive for some time in artificial surroundings was already recognized by Agassiz (1850), who kept his specimens alive by changing the sea-water twice a day. This author also gives a description of the structure of the polyps and states that they can be fed with fragments of molluscs. Besides this he gives some notes on the digestion in *Astrangia*. According to Agassiz the food at first remains for some time in the upper part of the gastric cavity before it descends to the lower part of this cavity where it is mixed with water and distributed over the internal organs of the polyp. As may be seen in the following pages these notes on the digestion are not completely in accordance with my observations.

The description of the general form of the polyps by Agassiz is very accurate. He states that there are three cycles of tentacles, those of the first cycle are of a larger size than those of the second, whilst the tentacles of the third cycle are somewhat smaller than the others. At each extremity of the laterally compressed mouth a tentacle of the first cycle is found. Agassiz discerned two varieties in the species: one with white polyps and another in which the polyps were pink or rosy.

An account of the chief peculiarities of *Astrangia danæ*, accompanied by figures, some of which represent the polyps in an expanded form, are found in the publications of Mrs. and Mr. Agassiz (1865) and of Dana (1890). Verrill and Smith (1874) also give a short description of the polyps of this species. They state that the animals are white and that the polyps in expansion rise high above the skeleton. These authors also note that the polyps will feed readily upon fragments of molluscs or crustaceans.

In the literature on the coral *Astrangia* I have not found any remark on its symbiosis with zoöxanthellæ. In the greater part of the colonies of this species found in the Woods Hole region all polyps are completely free of zoöxanthellæ. This was invariably the case in the specimens which I obtained from the piles of the wharf at Woods Hole, in those dredged in the harbour between Woods Hole and Nonamesset Island and in those dredged to the southeast of Nonamesset Island from about 8 fathoms. On the other hand the specimens dredged to the southwest of Falmouth (south of the oyster-pond) were always more or less infected with zoöxanthellæ. Some of the polyps of this locality had a dark brown color owing to the multitude of yellow algæ occurring in their tissues, others had various lighter shades of brown or were almost white, but a microscopical examination of a part of their tentacles or oral disk proved that they invariably contained zoöxanthellæ. It is a strange fact that this symbiosis of the polyp with yellow cells in the Woods Hole region is restricted only to the colonies found in certain smaller localities.

The polyps which are not infected by zoöxanthellæ are quite colorless and these are therefore especially fit for the study of the feeding reactions as foreign bodies can be seen through the transparent tissues of the animals. Usually the skeleton has a greenish or light-red color, which is due to the occurrence of algæ living in the skeleton. Then at first sight the polyps sometimes seem to have a pink or greenish hue by the transparency of their tissues. Probably these red or green-colored algæ are only different stages in the development of one species as their microscopical structure is very much alike. As yet I have no positive evidence that they belong to one of the forms described by Duerden (1905). In some colonies a compact mass of algal matter is obtained after

decalcifying the specimens, in other colonies only sparsely distributed threads are to be found. In an allied species, *Astrangia solitaria*, the skeleton is also penetrated by boring algæ (Duerden, 1902). Besides these boring forms there is another alga which lives in the tissue of the polyps. Mrs. Dr. A. Weber van Bosse, who kindly examined the preserved material of these algæ, found that it represents a new species of the genus *Streblonema*.[1]

For different reasons the polyps of *Astrangia* are especially fit objects for the study of their feeding habits. During the daytime they are usually fairly well expanded.[2] After being disturbed the polyps contract within the skeleton, but usually they will expand again in a very short time after the disturbance. Even when they are transferred from one glass-vessel to another they soon return to their expanded state. One of the further advantages of *Astrangia* in my experiments was that the polyps will ingest almost every particle which is offered to them, as well food as non-nutrient objects.

All the polyps of freshly-collected colonies of *Astrangia danæ* do not react in quite the same way on a certain stimulus, probably on account of the disturbance caused when they are collected. When, however, the colonies have been a few days in the laboratory under constant external influences they constitute a fairly uniform material for feeding experiments. Especially more or

[1] I am indebted to Mrs. Dr. A. Weber van Bosse for the following diagnosis of this interesting form:

"*Streblonema Willyae* n. sp.

Frondibus microscopicis in telum *Astrangiae danae* penetrantibus, compositis e filamentis sterilibus, irregulariter alterne aut secundatis ramosis, 2–5 $\mu$ latis, aggregatis, fasciculos prope superficiem hospitis formantibus. Chromatophoris taeniatis aut disciformibus, parietem cellulae non totius tegentibus. Sporangiis ignotis. Gametangiis cylindricis aut fusiformibus, singulis aut ramosis in filamentis plerumque terminalibus, interdum lateralibus, longis 60–120 $\mu$, latis 8–10 $\mu$; loculis uni- et pluriseriatis. Pilis desunt.

This new species of *Streblonema* is so far interesting as it grows in a coral, not in the skeleton but in the soft tissue of the animal. It has this mode of living in an animal in common with the genus *Endodictyon*, but the apical and above all the branched gametangia seem to indicate that its proper place is in the genus *Streblonema*."

[2] It is a well-known fact that the greater part of the madreporarian coral polyps are nearly always found in a contracted state during the day-time. Besides *Astrangia* there are, however, exceptions on this rule, *e.g.*, the polyps of *Fungia*, *Goniopora* and *Euphyllia* from the East Indian coral reefs, which are during daytime in a fully expanded state.

less hungry polyps are very susceptible to a certain stimulus, as is the rule in anthozoans. On the contrary well-fed polyps react very slowly on the same stimulus. Also in this respect *Astrangia* agrees with other anthozoans (cf. Jennings, 1905).

The chief results of my experiments on *Astrangia* are published in a preliminary paper (Boschma, 1925), in which especially the rôle of the zoöxanthellæ in the feeding of the polyps is compared with that in other anthozoans.

## Feeding Reactions.

The reactions of *Astrangia* to food and other substances are in many respects quite similar to those found in other representatives of the anthozoans. Before the results of my own investigations on *Astrangia* a short summary of the literature on this subject is given below.

Pollock (1883) was the first to describe that sea-anemones may perceive the presence of food-particles in their neighborhood, as this causes the opening of the mouth and movements of the tentacles of the animals. Loeb and Nagel some years afterwards worked out the reactions to food and other substances in actinians more in detail. According to Nagel (1892, 1894*a*, 1894*b*) the actinians have a sense of taste, which is located only in the tentacles, and the food is brought to the stomodæum by muscle-action. Nagel (1894*b*) already stated that hungry actinians also ingest morsels of paper besides food-particles, whereas well-fed ones will only take food. The results of Loeb (1891, 1895) differed in some respects from those of Nagel. The phenomenon which was called by Nagel the "sense of taste" is according to Loeb a reaction to chemical stimuli. Loeb showed that the tentacles are not the only organs in which the response to chemical stimuli is located, he stated that also ciliary actions plays a part in the feeding of actinians. Moreover by cutting an actinian into two halves Loeb proved that a part of such a polyp devoid of its tentacles even takes food more readily than a normal individual.

Parker (1896) confirmed Loeb's results that ciliary action is one of the chief factors in the feeding of actinians. Parker found that the cilia of the stomodæum generally beat outwards, but when food substances come into contact with these cilia they reverse

their movement. In subsequent papers (Parker, 1905*a*, 1905*b*) the same author has worked out this phenomenon in further details. He found that certain chemical substances can induce the cilia to reverse. A number of statements on the part of the tentacles and of the ciliary action in different actinians during the feeding reactions is described by Carlgren (1905). This author found that in some species of actinians the ingestion of food takes place chiefly by ciliary action (*e.g.* in the Protantheæ); in other forms, however, the tentacles are the chief organs for the capture and transportation of the food to the mouth (*e.g. Tealia*).

The fact that not all the individuals of a certain species of actinian react in the same way on the same stimulus, was demonstrated by Jennings (1905). The state of metabolism of the actinian is one of the most important factors determining the reaction to substances which come into contact with the tentacles or the oral disk. Thus hungry polyps often will ingest inedible matter, while well-fed ones will refuse the same material. Piéron (1906) also states that different individuals of one species behave differently towards the same stimulus.

Different species of actinians also behave in quite a different way. Some species of actinians in confinement would ingest only a very limited variety of food, *e.g. Eloactis*, which refused everything but living *Balanoglossus* and *Hydroides* (Hargitt, 1907). On the other hand many other species ingest besides food also indigestible material. Instances of this are recorded by Torrey (1905) for *Sagartia*, Fleure and Walton (1907) for *Tealia*, Parker (1896) and Allabach (1905) for *Metridium*. In the case of *Metridium*, Parker (1905), however, has expressed the opinion that the foreign matter (filter paper) was touched by the hands of the experimenter and therefore acted as a chemical stimulus. All of the above-named forms, as also *Actinia* (cf. van der Ghinst, 1906) are able to discern food-particles from inedible matter. The latter is usually refused and food particles are ingested.

Summarizing the data available in the literature and those of his own investigations Parker (1917) states that the different factors which are combined to bring about the feeding reactions in actinians are the following: secretion of mucus, ciliary action, the action of the neuromuscular apparatus of the tentacles, of the

œsophageal cilia, and of the transverse mesenteric muscles. Only the muscular reactions are under the control of the animal as a whole.

The feeding reactions of *Alcyonium* are described by Pratt (1906). In this form the tentacles are the chief organs for the capture of the food. The species exhibits a definite choice in selection of the food, as eggs of fish and of *Galathea* are not digested, whilst the polyps readily feed upon planktonic organisms and flesh of different fishes.

There are only a few papers dealing with the feeding reactions in madreporarian polyps. The first species, in which observations on the capture of the prey are recorded, is *Astrangia danæ.* Verrill and Smith (1874) mention that it catches its food with the tentacles, which afterwards transfer the food to the mouth. De Lacaze-Duthiers (1877) made some feeding experiments with *Caryophyllia Smithii* and *Balanophyllia regia.* In the former species the food, a piece of a living mollusk, placed on the oral disk, caused a depression of the disk in this place. By the action of the muscles of the oral disk it was now brought to the mouth. The tentacles remained quite inactive. In *Balanophyllia* on the contrary the tentacles pushed the food towards the mouth. After some time in both species the food was discharged through the mouth covered with mucus. These statements, however, can hardly represent the normal behavior of the polyps. Probably the animals were in an abnormal state, for De Lacaze-Duthiers had kept them during several years in captivity. Moreover these statements are not in accordance with those by Carlgren (1905) on *Caryophyllia.* According to Carlgren the tentacles of this species catch food-particles and deposit them on the central part of the oral disk. By ciliary movement they now are transported over the oral disk to the stomodæum and swallowed.

The polyps of *Siderastrea radians* seize the food, according to Duerden (1904), with the tentacles. According to the same author (Duerden, 1905) mucus plays an important part in the feeding reactions of *Fungia* and *Favia.* On the surface of the polyps a mucous layer is secreted. Small organisms which come into contact with the oral surface of the polyps are imbedded in the mucus, which is afterwards ingested.

Carpenter (1910) described the feeding reactions of *Isophyllia*. The tentacles of this species catch small planktonic organisms. When a certain amount of food is taken by the tentacles the sphincter of the edge-zone of the oral disk contracts and the oral disk then forms a kind of roof over the mouth and the surrounding parts. In the superficial chamber formed in this way the digestion of the food takes place.

Vaughan (1912, 1919) studied the reactions to food in *Mœandra areolata* and many other West Indian reef-corals. The food is ingested through the combined action of ciliary movements, secretion of mucus, and the movements of the tentacles. In some species, *e.g.*, in *Orbicella cavernosa*, the mesenterial filaments are often protruded through the column wall and can catch food and even digest it whilst remaining outside of the gastric cavity.

With my feeding experiments on *Astrangia* I could confirm the statements of former authors that it will readily take food in captivity. When not overfed the polyps even will ingest almost everything which is offered to them. The food-objects which I used in my experiments were the following: crab meat (the muscles of the legs of the spider crab, *Libinia caniculata* Say.), fish meat, the soft parts of mussels, different species of worms (*e.g.*, *Naraganseta coralii* Leidy which lives burrowing in the skeleton of *Astrangia*, *Hydroides* and other polychæt worms), pycnogonids, small amphipods, larvæ of higher crustaceans, copepods, ctenophores, etc. Also juice of crab or mussel meat is readily ingested. Besides these food-particles indigestible objects are ingested by hungry polyps with the same avidity: coarse sand, powdered writing chalk soaked in sea-water, clumps of litmus, carmine and iron carbonate.

When crab meat or fish meat is mixed with some coloring matter (India ink, litmus, ammoniac carmine, neutral red) it is as readily taken as pure meat. This method enabled me to trace the way of the food in the internal organs after it had been ingested, the coloring matter was only used as an indicator.

These experiments with pieces of meat could not give results which represent the normal feeding reactions. In the gastric cavity of freshly collected specimens often remains of small organisms are found, especially appendages of small crustaceans.

To study the normal feeding reactions I therefore fed the polyps with planktonic organisms, chiefly copepods and larvæ of decapod crustaceans. It proved to be an advantage when the food was colored, then it could be seen through the transparent tissues when it was captured and swallowed. For this reason I used the method employed by Fischel (1908) for daphnids and copepods of fresh water. To the sea-water which contained the animals a few drops of a solution of neutral red were added by means of which the water obtained a light yellow color. The copepods and other organisms now absorbed the coloring matter and gradually acquired a bright red hue. Especially the different parts of the intestinal tract absorbed a great quantity of neutral red, but also immediately beneath the skeleton and in the appendages the coloring matter was stored. These colored copepods and larvæ of decapods are as readily taken by the polyps as colorless ones.

When some of these colored planktonic organisms are put into a small glass containing a colony of *Astrangia* with expanded polyps the capture of these animals may be easily observed. Every now and then one of the animals comes into contact with a tentacle of a polyp. Smaller copepods then as a rule are immediately captured, they seem to stick to the tentacle, undoubtedly by their being paralyzed by nematocysts. The tentacle then suddenly contracts more or less and bends over the oral disk in the direction of the mouth. The prey, however, is not brought directly to the mouth. The latter, with the central part of the oral disk, slowly increases in height as a conical protusion and this expanded part gradually moves in the direction of the prey. At last the tentacle with the copepod comes into contact with the mouth, the tentacle releases the prey and bends back to its original place. The captured copepod slowly slides down through the stomodæum into the gastric cavity, undoubtedly by ciliary action, and the mouth returns to the central part of the disk.

Larger copepods and larvæ of decapods, however, are not so easily captured. When they touch a tentacle they often swim away with a sudden jerk, the attack of the nematocysts seeming insufficient to paralyze them. Only when these animals bump heavily against a tentacle they are immediately caught. Their

struggling movements cause a number of neighboring tentacles to move towards them, so that they finally become completely enveloped in a number of tentacles. Then they are slowly transferred to the mouth in the above described way, but they often remain struggling heavily, even when going down the stomodæum. Usually, however, the tentacles keep these larger organisms in the same position for some time, and only after they have ceased to struggle they are transferred to the mouth.

These experiments with copepods and other small crustaceans show that the tentacles instantly react when they are touched by a free swimming animal. The oral disk on the contrary is quite indifferent to the contact of these planktonic organisms. Sometimes a small floating copepod may be seen sinking down and falling on the oral disk of a coral polyp. It can remain there for some time without calling forth any reaction of the polyp. Afterwards it can swim away unharmed unless it happens to touch one of the tentacles, in which case it immediately brings forth the capturing reactions of the tentacle.

In the case of the feeding experiments with crab meat or other non-moving material of food the movements of the tentacles are very slow. In expanded polyps the small morsels of meat strongly adhere to the tentacles to which it is offered, and usually some neighboring tentacles also bend towards the meat, more or less enveloping it. The mouth with the top of the conically expanded central part of the oral disk moves slowly towards the prey and the tentacles push the meat downwards into the stomodæum. Some tentacles often protrude into the stomodæum, pushing against the food-material. The mouth then closes and the food slowly passes down into the gastric cavity.

Generally after the feeding the polyps partly expand by raising their column often considerably above the skeleton. The tentacles also remain in a more or less expanded condition. This expansion of the polyps generally takes place as well after the feeding of meat as after the feeding of free-swimming organisms. In this state the polyps are almost indifferent to mechanical stimuli. Whilst a hungry polyp quickly contracts when gently touched by a forceps the polyps which have just fed do not react on the same stimulus. A further peculiarity is that often some

time after feeding air-bubbles are to be seen in the upper parts of the polyps, probably escaping from the prey which is being dissolved in the gastric cavity.

The reactions of hungry polyps of *Astrangia* to comparatively heavy objects, as diminutive pebbles and clumps of litmus, writing chalk or iron carbonate, are almost the same. These objects, when laid against a tentacle or on the oral disk, bring about the feeding reaction, consisting of the above described movements of the tentacles and the central part of the oral disk. The objects are almost invariably ingested and come closely in contact with the mesenterial filaments. The mouth closes after the ingestion and the polyp remains for some time, in my experiments varying between 20 and 95 minutes (usually lasting about 50 minutes), quite motionless. The first movement made after this time usually is the enlargement of the central part of the oral disk to a conical protusion. Then the mouth opens and the foreign object is seen moving slowly upwards in the gastric cavity. The underside of the object is in touch with the mesenterial filaments of the polyp, which seem to push against it. In the stomodæum in all probability the outward movement of the foreign object is caused by ciliary action, but often some mesenterial filaments are seen protruding in the stomadæum till the object has been removed from the inside of the polyp. In this way the non-nutrient paticles are soon out of the gastric cavity, chiefly by the action of the mesenterial filaments. The object falls from the mouth on the oral disk and slides down on one side. Here the tentacles bend downwards and then the object is completely removed from the polyp.

The foreign objects which have been in the gastric cavity for some time are covered with a thin mucous layer. Undoubtedly the mucus has here a protective function, to avoid that noxious particles come into contact with the tissues of the polyp. This function of the mucus is already described by Gee (1913) in *Cribrina*. In *Astrangia* especially on the clumps of chalk which are removed from the gastric cavity the mucus is clearly visible. Often other particles imbedded in this layer are removed with the non-nutrient object. In this way very small quantities of remains of food may be obtained from the gastric cavity without

causing any injury to the polyp. I used this method to make out whether or not zoöxanthellæ were present in the gastric cavity of polyps the tissues of which contained these unicellular algæ.

Small pieces of filter paper rarely induce the polyps of *Astrangia* to feeding reactions. To avoid the absorption of organic substances (cf. Parker, 1905) the pieces of paper were not touched with the hands, but they were handled by clean instruments and put on the tentacles of a hungry polyp. For some time the small objects remain attached to the tentacles, to which they adhere more or less, but as a rule they soon fall down without causing the feeding reaction. In some cases, however, a few pieces of filter paper are ingested and afterwards, after about 50 minutes, removed from the gastric cavity in the above described manner. The difference in behavior of hungry polyps to comparatively heavy objects as small pebbles and pieces of filter paper in all probability is caused by the difference in weight. The heavy objects give a definite mechanical stimulus which immediately brings forth the feeding reaction, whilst the pieces of filter paper act as indifferent objects.

When carmine-powder soaked in seawater is distributed over expanded hungry polyps large quantities are ingested. There is no evidence of ciliary action on the oral disk, probably cilia are not to be found here. Soon after the carmine has dropped on the oral disk it is imbedded in mucus and large strains of this mixture of mucus and carmine can be seen gliding down the stomodæum through the inward beating of the cilia of the latter. Often a quantity of carmine is ingested by the cells of the mesenterial filaments, but it also occurs, especially when large quantities of carmine are present in the gastric cavity, that clumps of carmine mixed with mucus are removed from this cavity as non-nutrient particles.

The feeding reactions of *Astrangia* in general therefore consist chiefly of four actions of the polyps: muscular action of the tentacles, muscular action of the central part of the oral disk, secretion of mucus by the oral disk, and ciliary action of the stomodæum.

## Digestion.

In a great number of anthozoans, chiefly in actinians, the phenomena of digestion are at least partially investigated. The different authors who have worked on this subject do not agree in every detail, though the chief features of the digestion are fairly well known.

The principles of our knowledge of the phenomena of digestion in actinians and other cœlenterates are chiefly due to Metschnikoff and Krukenberg. Metschnikoff (1880, 1882) found that small food-particles are ingested by the entoderm cells of the mesenterial filaments in an amœboid way. They are imbedded in these cells and digested here (intracellular digestion). Claus (1881) maintained that the ingestion of foreign corpuscles in the mesenterial filaments of cœlenterates was already described by him before Metschnikoff made mention of this fact. But in Metschnikoff's papers the phenomenon of ingestion was described in quite a convincing form, whilst Claus' previous investigations (Claus, 1874) only contained some notes on the question.

Krukenberg (1880) proved that the mesenterial filaments of actinians are organs of digestion and maintained that digestion takes place solely against these organs. According to this author the mesenterial filaments have to come in contact with the food, then a secretion of digestive fluid takes place and the food-particles are more or less dissolved (extracellular digestion)[3]. Krukenberg (1881, 1882*a*, 1882–86) was not inclined to attribute such an important function to the intracellular digestion of small particles as described by Metschnikoff. He was convinced that the filaments secrete a digestive fluid, though he was not able to demonstrate this fluid in a free state in the gastric cavity. He even expressed the opinion that the enzymes of the captured animals could be used by the actinians for their digestion (Krukenberg 1882*b*, 1882*c*).

The investigations of Willem (1892, 1893) made it probably that besides intracellular digestion also extracellular digestion of

[3] In the work of O. and R. Hertwig (1879/80) already the opinion is put forward that the mesenterial filaments secrete a digestive fluid. This was based only on the study of the histological structure of these organs. The definite proof of the digestive function of the mesenterial filaments is given by the physiological studies of Metschnikoff and Krukenberg.

the food takes place in actinians. The larger food-particles are more or less dissolved into smaller corpuscles by the secretion of a digestive fluid, and afterwards the small objects are ingested by the entoderm-cells as described by Metschnikoff. The ingestion of these small particles usually takes place in a zone of the mesenterial filaments in the neighborhood of the free edge, but after abundant feeding all the entoderm cells of the gastric cavity can ingest food, even those of the acontia. In the sea-anemone *Tealia* the soft parts of amphipods of the genus *Talitrus* are completely dissolved till only the bare skeleton is left (Willem, 1892). In siphonophores Willem (1894) found approximately the same phenomena: here also extracellular and intracellular digestion are present.

According to Chapeaux (1893) in the gastric cavity of actinians a free digestive fluid can be demonstrated. The secretion of this fluid occurs when food comes into contact with the mesenterial filaments. When the food is ingested in the entoderm cells the reactions of the food-vacuoles is acid. In siphonophores Chapeaux found that 15–20 hours after ingestion of food colored with litmus the vacuoles still had a red color. Chapeaux concluded from this fact that the digestive enzymes of actinians and siphonophores acted in an acid medium. The same opinion also is upheld by Metschnikoff (1893), Mesnil (1909) and Roaf (1910). On the other hand Jordan (1907*a*) supposed that after the acid reaction in the vacuoles an alkaline one would follow; the final resorption of the food probably would take place in the latter stage. The enzymes of the anthozoans then would present more likeness to those of other groups of invertebrates, and act in about the same way as the trypsin of vertebrates (cf. also Jordan, 1907*b*).

A number of investigations on digestion in actinians have been made to show whether there is only intracellular digestion in these animals or if there also is secreted a free digestive fluid which dissolves the larger corpuscles into small particles which can be ingested by the entoderm cells. According to Mesnil (1901) no free enzyme is secreted in the gastric cavity: the digestion in actinians is exclusively intracellular. Jordan (1907*a*), however, showed that food which could not come into contact with the

mesenterial filaments could be digested, and concluded that an enzyme causing extracellular digestion can be secreted by the mesenterial filaments. This opinion is also found in an article by Willem (1916), who maintains that extracellular digestion of the larger particles precedes the ingestion (phagocytosis) of the small particles, which are further dissolved by intracellular digestion.

The opinion of Biedermann (1911) on this question is in some respects an intermediate one between the two views dealt with above: he supposes that besides the intracellular digestion also the secretion of a digestive enzyme occurs. The reason why the enzyme is not found free in the gastric cavity (Mesnil, 1901) is according to Biedermann probably the following: only small quantities of digestive fluid are secreted by the mesenterial filaments, and only in immediate contact with the food.

Jordan (1913) upholds his original opinion, based on experiments with food packed in filter paper (Jordan, 1907*a*), which was digested in the gastric cavity of the polyps. This proves that the enzyme can easily penetrate through the whole of the food corpuscles and diffuse through every part of the gastric cavity where food particles are present. There is no special need for a contact of the food with the mesenterial filaments.

The principal organs of digestion in madreporarians are the mesenterial filaments, the same as in actinians. In *Cœnopsammia* (*Dendrophyllia*) Gardiner (1900) found a small crustacean in one of the polyps, supported by the mesenterial filaments, which indicates that it was being digested here. In the mesenterial filaments of *Flabellum* the same author (Gardiner, 1904*a*) found fat globules and diatoms and other algal matter in the cells of the portion next to the edge which is crowded with nematocysts. When remains of partially digested food were found in the gastric cavity of madreporian polyps, these were lying against the mesenterial filaments, as recorded by Carpenter (1910) for *Isophyllia* (*Mussa*) and by myself (1924) for *Favia*. Moreover strong evidence for the digestive function of the mesenterial filaments is given by Vaughan (1912), who states that these organs in *Orbicella* may protrude through the column wall and catch and digest the food outside the gastric cavity.

In other groups of Anthozoa there are also a number of statements on the digestive function of the mesenterial filaments. In *Pennatula* and *Virgularia* Marshall and Marshall (1882) found foreign bodies embedded in the cells of these organs. Wilson (1883) has observed the ingestion of food by the mesenterial filaments of *Leptogorgia*, and the same author found diatoms and other solid foreign corpuscles enclosed in the mesenterial filaments of alcyonid polyps (Wilson, 1884). The most elaborate researches on digestion in octactinians have been made on *Alcyonium*. According to Hickson (1901) the mesenterial filaments of this animal secrete a digestive fluid which dissolves the food. The latter is afterwards ingested by the entoderm of the gastric cavity. These conclusions were confirmed by Pratt (1906). After elaborate feeding experiments on *Alcyonium* and the study of the changes in histological structure before and after feeding in the polyps of this form Pratt came to the same conclusion: in alcyonid polyps extracellular as well as intracellular digestion occurs. Before feeding the gland cells in the stomodæum and in the mesenterial filaments are filled with a secretion, after feeding they are empty. Pratt concludes that this secretion is mixed with the food in the gastric cavity and causes the partial dissolution of the larger objects. Afterwards the small particles are ingested by the cells of the mesenterial filaments.

The opinion of Dantan (1921) that the digestion of antipatharian polyps is only extracellular is solely based on the histological study of the one polyp in which food-particles could be found. This observation does not give sufficient evidence for the opinion that the polyps of this group are in respect to their digestion quite different from all other anthozoans.

In the mesenterial filaments of zoanthids also foreign bodies are found embedded in the cells (McMurrich, 1889, 1899). The zoanthid polyps therefore in all probability digest their food in the same way as other anthozoans.

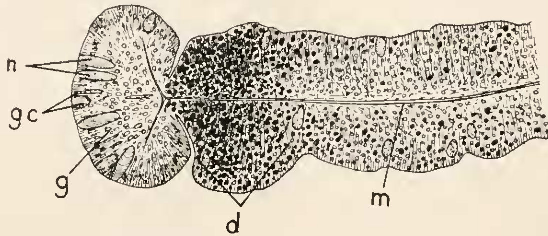
As stated above, a great number of investigators found that the mesenterial filaments are the chief organs of digestion in anthozoans. In many of these statements the accurate place of the ingestion is said to be the part immediately behind the marginal zone which contains a great number of gland cells and nemato-

cysts. The mesenterial filament then consists of a marginal, glandular part and a part with digestive function next to the marginal part. Morphologically, however, the word "mesenterial filament" usually is employed in a more restricted sense, meaning only the marginal, glandular part (cf. Schneider, 1902). Also Duerden (1902), who states that the swollen part of the mesentery next to the marginal region is generally considered to be the principal seat of digestive function, denotes the marginal region only as the mesenterial filament. I use here the term in the same sense as other authors who studied digestion in anthozoans, and differently from the definitions of Schneider and Duerden. The glandular part of the mesenterial filament contains the bifurcation of the mesoglœa. It is sharply separated from the digestive part by a deep furrow; the digestive part is, as in other madreporarians (cf. Duerden, 1902), not sharply separated from the rest of the mesentery, usually its thickness is slightly more.

In my studies on the digestion of *Astrangia* I chiefly used pounded crab meat thoroughly mixed with some coloring matter which served as an indicator after the food had been swallowed. A number of small colonies served as material for each experiment. I noted the time of feeding and studied the changes in the internal organs at various intervals by cutting open a polyp and examining a mesenterial filament under the microscope.

In the case of crab meat colored with India ink the food is readily taken by the tentacles and transferred to the mouth. Then it slowly descends through the stomodæum into the gastric cavity. By the transparency of the tissues it remains visible in the lower part of this cavity, where it is in contact with the mesenterial filaments. The polyps gradually expand more or less and often rise considerably above the skeleton (to about 15 mm., the tentacles also may stretch to the same length). When, after a very short time, the food is ingested into the mesenterial filaments these organs are visible as dark stripes through the wall of the polyps. Already one hour after feeding the digestive part of the mesenterial filament is crowded with small black spots (the particles of India ink) which indicate that the food with the coloring matter has been ingested here. Sparsely distributed black particles are also often found in the adjoining portion of the

mesentery. When food in great abundance has been offered to the polyps these parts ingest a great deal of it; when only moderate portions of food are given nearly all the food is ingested in the zone next to the marginal part. During a few days the distribution of the black particles in the mesenterial filaments does not alter perceptibly (Fig. 1); afterwards (about 5 days after




EXPLANATION OF THE TEXT FIGURE.

Transverse section of the free extremity of a mesentery of *Astrangia danæ*, circa 2 days after the feeding of the polyp with a mixture of crab meat and India ink. Fixation 5 per cent. trichloracetic acid; stained with hematoxylin Delafield, *d*, digestive region of the mesenterial filament, crowded with black particles; *g*. glandular part of the mesenterial filament; *gc*, gland cells; *m*, mesoglœa; *n*, nematocysts. × 360.

feeding) the black particles gradually disappear from the digestive part of the mesenterial filaments. On the seventh day after the feeding usually the black particles have completely vanished from the digestive organs.

Crab meat mixed with ammonia carmine gave approximately the same results: during a number of days after the feeding the digestive zone of the mesenterial filaments then contains a quantity of red vacuoles.

Another series of feeding experiments was made with crab meat mixed with litmus. This mixture keeps its blue color whilst remaining in the gastric cavity, but after the food has been ingested in the mesenterial filaments the latter acquire a red color. This change of color is clearly visible in the living animal through its transparent tissues. Microscopic examination of a mesenterial filament soon after feeding proves that in the digestive zone a large number of red vacuoles of different size are present, whilst particles of litmus lying against the mesenterial filaments but not yet ingested have retained their blue color. Consequently the

digestive vacuoles possess an acid reaction whereas in the gastric cavity this reaction is neutral (or weakly alkaline).

During two days the digestive vacuoles keep approximately the same color. After about 48 hours a few vacuoles have acquired a bluish hue. Gradually the number of the blue vacuoles increases and after about three days the majority of the vacuoles in the digestive zone of the mesenterial filaments are blue. At last there are only a few red spots left, whilst the great number of blue vacuoles remain till about seven days after the feeding. During the greater part of the time the color is evenly distributed in the comparatively large vacuoles, but towards the end of the alkaline period (on the sixth and seventh day after the feeding) the coloring matter is concentrated to small particles, which gradually disappear from the mesenterial filaments.

In the digestion of *Astrangia* we can therefore distinguish two periods: an acid one, lasting for about two days, and an alkaline period during the rest of the time. In the later part of this alkaline period the excretion of the undigestible particles occurs. The changes in the reaction of the digestive vacuoles make it highly probable that the absorption of the food takes place in the alkaline period only. The acid period then is an antiseptic one (cf. Jordan, 1907*b*), in which noxious microörganisms are killed. The authors who stated that digestion of actinians takes place in an acid medium (cf. Chapeaux, Mesnil) did not extend their experiments for a sufficient time, otherwise they probably would have found that also in actinians the acid reaction is followed by an alkaline one. As compared with protozoans these two periods last a very long time. In *Paramecium* and *Colpidium* after feeding there is an acid period of $5\frac{1}{2}$ to 70 minutes which is followed by an alkaline period of 1 to 30 minutes (Nirenstein, 1905).

One of the arguments for the presence of extracellular digestion in actinians results from the experiments of Willem (1892) referred to above. In *Astrangia* I have employed approximately the same method to demonstrate the evidence for extracellular digestion. I therefore studied the digestion of large copepods by the polyps. The copepods were vitally stained with neutral red after Fischel's method. At various intervals after the feeding I

opened a polyp and noted the changes in the mesenterial filaments and in the copepods. The results were the following.

Half an hour after the feeding in the digestive zone of the mesenterial filaments a number of red vacuoles are visible. Besides these vacuoles which have a fuchsin like hue (acid reaction), the remainder of the mesenterial filament, including the marginal part, has absorbed some neutral red which is unchanged in color. The copepod still has a deep red hue.

Gradually now the intensity of the color of the mesenterial filaments increases whilst the color (together with the muscles and other soft parts) is extracted from the copepod. At first the parts of the copepod which are lying against the mesenterial filaments lose their color, the distal parts of the appendages usually keep the neutral red longer than the central part of the animal. After two hours the mesenterial filaments are strongly colored, whilst the copepod is almost completely devoid of its soft parts and only the bare skeleton is left.

When the polyps are cut open to study the changes in the food and in the digestive organs the parts of the skeleton of the copepod usually disjoint. To avoid this the experiment can be modified in some respects. When a polyp, including the skeleton, is cut longitudinally into two halves, the digestive function of the mesenterial filaments may be studied under the microscope. The skeleton prevents the tissues from contracting strongly after the dissection and the mesenterial filaments remain clearly visible.

A copepod vitally stained with neutral red placed on the mesenterial filaments soon becomes partly enveloped by a few coils of these organs and the struggling movements of the animal soon come to an end. After some time (a quarter of an hour to half an hour) these coils withdraw from the food and then the digestive zone of their surface which has been in contact with the copepod has assumed a red color, thereby forming a striking contrast with the remaining parts of the mesenterial filaments. These colored parts, then apparently saturated with food, slowly bend away from the copepod and other coils of the same or a neighboring mesenterial filament take their place. The copepod remains intact as far as the external form is concerned till almost all of the colored internal tissues have been absorbed by the mesenterial

filaments. Afterwards usually the skeleton falls into pieces but not before almost the whole of the internal organs are dissolved. After about three hours the copepod is nearly completely devoid of its soft parts and the more or less disjointed skeletal elements are removed from between the mesenterial filaments.

This experiment, which can be easily followed under the microscope from the beginning till the end, gives a strong evidence for the view that besides intracellular digestion there is in *Astrangia* a secretion of an enzyme which dissolves the food in an extracellular way. Whilst the external surface of the copepod still is completely intact the greater part of the internal organs are already dissolved and ingested by the cells of the digestive zone of the mesenterial filaments. This disintegration of the soft parts of the copepod, as long as it does not yet fall into pieces, is only possible when a digestive fluid penetrates into it.

Probably in *Astrangia* a number of different digestive enzymes may be demonstrated as in the case of other cœlenterates (cf. Bodansky and Rose, 1922). The most effective of these enzymes is undoubtedly one which is comparable to the trypsin of other animals. This we may already expect in advance as the digestive vacuoles during the later period have an alkaline reaction. Moreover in all lower animals in which the proteolytic enzymes are studied they have a trypsin-like function (cf. Jordan, 1907*b*). I have made no elaborate experiments on the nature of the enzymes in *Astrangia*, but the few enzyme preparations tested showed that a trypsin-like enzyme is the chief factor for the disintegration of the food. Owing to the polyps being small the mesenterial filaments cannot easily be separated from the other parts of the polyps. The suspensions were made by pounding the tissues (chiefly consisting of the mesenterial filaments, extracted from a number of polyps) with sand to a mash. The latter was diluted with sea water and preserved with a few drops of chloroform. To equal parts of tissue suspension a piece of crab meat (previously boiled to destroy the blood enzymes of the crab it might contain) was added. Tests, containing crab meat in sea water with chloroform, were prepared in the same time. After four days the meat was partially dissolved. No positive results were obtained with the biuret reaction; the reaction with ninhydrin,

however, gave a definite purplish blue color, proving that the liquid contained amino-acids or peptids (cf. Howell, 1922). In the tests the crab meat was practically unaltered, the liquid gave only negative results with both reagents.

## The Food of Astrangia and the Symbiotic Algæ.

Among my material there were a great number of colonies the polyps of which possessed numerous zoöxanthellæ in their entoderm. The feeding of these polyps therefore can to some extent be compared with that of reef-corals, which, as a rule, also contain large quantities of these unicellular algæ. On the other hand the polyps of *Astrangia* with the symbiotic organisms can be directly compared with those of the same species which are completely free of zoöxanthellæ.

In the literature there are comperatively few notes on the food of Madreporaria. According to the statements of many authors (cf. Duerden, 1902; Gardiner, 1902–03; Pratt, 1906; Walther, 1919) only very rarely remnants of food are found in the gastric cavity of madreporarian and also of alcyonarian polyps. Now the greater part of the researches on which these statements are based were made after the study of preserved material, and as I have already pointed out before (Boschma, 1924) this is at least partially due to the contraction of the polyps in the fixing fluid. When coral-polyps strongly contract the food-remnants which are in their gastric cavity usually are discharged through the mouth.[4] In living coral-polyps from the reefs in the East Indian region I usually found remnants of food in the gastric cavity embedded in mucus.

To ascertain the natural food of *Astrangia* I now proceeded in the same way and studied the specimens in the living state. As soon as the colonies were dredged I put them in sea-water on board the ship and left them undisturbed for about a quarter of an hour. Gradually the polyps now expanded more or less. After a mechanical stimulus (a slight touch with a forceps) they suddenly contracted, and at the same time the mouth opened widely. Usually then at the bottom of the gastric cavity a slight

[4] Some years ago Dantan (1921) also tried to explain this lack of food in the gastric cavity of anthozoans by the action of the preserving fluids which cause contraction and emptying of the gastric cavity.

amount of foreign material was to be seen, which could easily be extracted with a small forceps, without any damage to the polyps. The contents of the gastric cavity of 20 polyps consisted besides of undeterminable matter (detritus) of the following foreign organisms or parts of these: living diatoms (found in 12 polyps), diatom scales (found in 9 polyps), parts of higher algæ, usually in a partially decayed state (found in 4 polyps), foraminifera (found in 1 polyp), spicules of sponges (found in 5 polyps), parts of the stalks of hydroids (found in 2 polyps), a living nematode (found in 1 polyp), a dead larva of a polychæt worm (found in 1 polyp), parts of appendages or segments of the body of different smaller crustaceans (found in 10 polyps), shells of small bivalve mollusks (found in 3 polyps). Often also nematocysts or parts of these occur among the food-remnants and in the polyps which live in symbiosis with zoöxanthellæ invariably also these yellow algæ are to be found in the gastric cavity. Only very few polyps, when examined immediately after being collected, do not contain anything in their gastric cavity.

It is an interesting fact that in those polyps of *Astrangia* in which zoöxanthellæ occur in the entoderm cells, these algæ are always found in the remains of the food in the gastric cavity. These algæ are here in different stages of decomposition, owing to their being digested by the polyps. In this respect the polyps of *Astrangia* possessing zoöxanthellæ agree closely with reef-corals, in which the symbiotic algæ are also found in a partially digested state in the mesenterial filaments.[5]

There are, in general, two opinions concerning the food of reef-corals. One of these opinions was first put forward in a number of publications by Gardiner (1899, 1902, 1902–03, 1904*b*). This author found that the zoöxanthellæ form a large proportion of the food of all reef-corals, and maintains that many species of these corals even feed entirely on their symbiotic algæ. In a later publication Gardiner (1912) states that zoöxanthellæ are largely eaten by the coral-polyps when they require food, and further mentions that it is supposed that they catch and digest the small

[5] After my studies in Woods Hole I made some observations on the feeding of a few reef corals in the Bermuda Biological Station for Research. The polyps of these corals (*Isophyllia* and *Siderastrea*) in the natural state invariably contain partially decayed zoöxanthellæ in the digestive zone of the mesenterial filaments.

organisms of the superficial water of the sea. Hickson (1906) admits that the zoöxanthellæ may constitute a part of the food of reef-corals, but thinks it improbable that there are coral-polyps that feed exclusively on their yellow cells. In a recent work of Hickson the opinion is upheld that probably "the holozoic method of nutrition of the coral is supplemented by the holophytic action of the chlorophyll-bearing zoöxanthellæ" (Hickson, 1924, p. 21).

The other opinion on the food of reef-corals is expressed by Vaughan (1912, 1919) after a great number of feeding experiments on West Indian reef-corals. His conclusion is that the food of reef-corals solely consists of animal matter. This view is also supported by Mayer (1918), who based his opinion on Vaughan's experiments and on the statement of Duerden (1904) that the polyps of *Siderastrea* are easily kept alive with meat of crabs and other animals.[6]

Vaughan's opinion was first criticized by Gravier (1913). According to Gravier it is improbable that coral-polyps live exclusively on animal matter, for their symbiotic algæ are undoubtedly also a factor in their nutrition as direct food or indirectly by the supply of carbohydrates. After the study of the contents of the cœlenteron of many coral-polyps I also (Boschma, 1924) came to a conclusion differing from Vaughan's. I found that the food-remnants in the gastric cavity of the polyps of reef-corals always contained zoöxanthellæ in various stages of disintegration, undoubtedly owing to their being digested by the polyps. Besides these algæ also animal matter was found in the remains of the food, rarely in the smaller polyps, more often in the larger polyps.

It is generally understood that the products of the photosynthesis of the zoöxanthellæ assist in the nutrition of the cœlenterates containing these algæ (Buchner 1921, 1924; Hickson, 1924). In many anthozoans which harbor large quantities of these unicellular algæ even a degeneration of the food-capturing

[6] Besides the facts on the food of reef corals there are some statements on the food of madreporarian corals which do not contain zoöxanthellæ in their entoderm. Some notes on the food of deep-sea corals are found in the publications of Gravier (1920) and Boschma (1924). In the latter article also data on the food of species of *Dendrophyllia* from shallow water are given. Cf. also Gardiner's articles on *Cœnopsammia* and *Flabellum*, cited above.

and digestive portions of the polyps has taken place, as in *Sclerophytum gardineri* (Pratt, 1903, 1906) and in *Galaxea musicalis* (Matthai, 1914).

The feeding of cœlenterates on their symbiotic algæ as a whole has been reported in a few cases only. In young medusæ of *Aurelia aurita* Friedemann (1902) has mentioned instances of this phenomenon. According to Pratt (1903) in the polyps of the alcyonarian coral *Sclerophytum* frequently zoöxanthellæ are observed in a partially digested condition in the mesenterial filaments (cf. also Pratt, 1906). Digestion of zoöxanthellæ in the polyps of reef corals is recorded by myself (1924). Probably the irregular green corpuscles which McMurrich (1889) found among the zoöxanthellæ in the digestive part of the mesenterial filaments of zoanthids were also such partially digested algæ. In *Velella* the zoöxanthellæ of the developing larvæ which migrate to deeper water probably serve as a source of food-substance only, as their photosynthetic action is impeded by the darkness (Woltereck, 1904).

According to Fulton (1921, 1922) the association of cœlenterates with their zoöxanthellæ probably is of a parasitic nature (the polyps being the parasites of the algæ), as during starvation sea-anemones feed upon the unicellular algæ rather than upon their photosynthetic products.

Besides their feeding on the zoöxanthellæ or on their products of assimilation the polyps also derive some profit from the algæ living in their tissues, as they are a source of oxygen for the polyps. The investigations of Brandt (1883), Trendelenburg (1908) and Pütter (1911) prove that actinians with zoöxanthellæ may derive a large part of their oxygen from these algæ, and that actinians with zoöxanthellæ can better resist unfavorable circumstances than those which do not harbor unicellular algæ. Probably the algæ also use nitrogenous waste products of the polyps, which may be one of the profits the zoöxanthellæ derive from the association with the cœlenterates.

In the case of *Astrangia* zoöxanthellæ are always found in the digestive region of the mesenterial filaments of the polyps which are infected with these algæ. In contradistinction to the algæ in the entoderm of the oral disk and the tentacles those in the

digestive zone have lost their yellow color to some extent. White spots then appear in their interior, whilst the contents are contracted and often have assumed a brownish hue. All different stages of the decomposition of these algæ are found in the digestive region, indicating the probability that they are being digested here. In the polyps of *Astrangia* which are not infected with zoöxanthellæ these algæ never occur in the digestive region of the mesenterial filaments. Consequently the zoöxanthellæ do not form a necessary part in the feeding of the polyps. A priori it is highly probable that they are digested on account of the lack of other food. We may therefore expect that, when food of other origin in great abundance is given to the polyps, the cells of the digestive zone of the mesenterial filaments will ingest no more zoöxanthellæ.

In fact these changes of the contents of the digestive region are easily accomplished. For my experiments I used dark brown polyps, the digestive organs of which before the artificial feeding contained a large quantity of partially decayed zoöxanthellæ. These polyps now were repeatedly fed with crab meat and already after three days the digestive region of the mesenterial filaments was almost completely devoid of zoöxanthellæ. Still a few yellow-brown corpuscles were recognizable as strongly decayed algæ, but it was evident that after the feeding of the polyps with crab meat no more algæ were ingested. The zoöxanthellæ which were already present in the digestive organs were completely digested. In this way the polyps can be compelled to change the nature of their food. It is obvious from these facts that the zoöxanthellæ are ingested by comparatively hungry polyps only. In the natural state the amount of food available for the polyps seems to be rather scanty and therefore they usually derive a part of their food from the digested unicellular algæ.

Van Trigt (1919) has described a similar phenomenon in freshwater sponges (*Spongilla*), which contain zoöchlorellæ. These unicellular algæ, which are continually imported from the surrounding water, constitute a very important source of food for the sponges. When circumstances are favorable the algæ are killed and digested by the sponge only in part, the rest of the imported algæ then can live on, photosynthesize and multiply in the tissues.

On the other hand in less favorable circumstances the whole of the imported algæ together with those already present in the tissues of the sponges are digested.

### Infection of Colorless Polyps with Zoöxanthellæ.

In my experiments I kept the colonies of white polyps separated from those with brown polyps (brown on account of their zoöxanthellæ). As the white polyps did not become infected during a month's time there is little evidence that infection occurs through free zoöxanthellæ in the sea water. In a number of polyps I produced an artificial infection with zoöxanthellæ by feeding them with portions of the soft parts of dark brown polyps. Especially those parts of the brown polyps which contained a multitude of these algæ (the tentacles and the oral disk) were used for this experiment. The tissues were torn to minute pieces and thoroughly mixed with pounded crab meat. It was necessary to add meat to the tissue preparations for otherwise the polyps refused to feed upon it.

Soon after feeding a number of zoöxanthellæ had detached from the crab meat and were floating free in the gastric cavity of the polyps and sometimes even the tentacles assumed a light brown color by the algæ which had penetrated into their cavity. A large part of the zoöxanthellæ from the feeding mixture were ingested together with the meat by the mesenterial filaments. After three days the digestive region of these organs was crowded with algæ. Some of these were already partially digested, for white spots had appeared in the originally evenly yellow colored interior.

Some of the cells which a very short time after the feeding are found in the cavity of the tentacles penetrate into the entoderm cells of these organs. Here they quickly multiply by division into two halves. The following numbers clearly illustrate the quick increase in number of the zoöxanthellæ in the tentacles of recently infected polyps. In one polyp on the day after the infection a cut-off top of a tentacle contained 6 symbiotic algæ, three days after the infection a top of a tentacle of the same length contained 23. For another polyp the number of zoöxanthellæ in the entoderm of different distal parts of tentacles of about equal

size was: 1 day after the feeding 4 and 5; 2 days after the feeding 9, 16, 2, 21 and 12. In a third polyp the number of the algæ in tentacular extremities of approximately equal size was: 3 days after the feeding 30, 28, 22, 17 and 27; 9 days after the feeding 72 and 53. Consequently the zoöxanthellæ in the entoderm cells after the infection gradually had increased in number, though the change in color of the polyp could not yet be observed at first sight, for in moderately brown polyps extremities of tentacles of the same size as those cut-off in my experiments contain far more than 1,000 zoöxanthellæ.

After the feeding with the mixture of crab meat and zoöxanthellæ the infected polyps were fed several times with crab meat to keep them in a well-fed state. In hungry polyps the zoöxanthellæ perhaps might divide less rapidly.

I could not continue these experiments of infection for more than 9 days, but I am convinced that in this way a white polyp may be changed into a brown one crowded with zoöxanthellæ in the entoderm cells. Probably in nature the infection may take place (though rarely) in a similar way. When the polyps capture an animal which contains zoöxanthellæ some of these may find their way to the entoderm of the tentacles or the oral disk. After division these cells may infect the neighboring cells and at last the whole of the entoderm of the colony. This manner of infection in all probability only rarely occurs. As in other coral polyps with symbiotic algæ the greater part of the infected polyps of *Astrangia* undoubtedly have originated from planulæ which already obtained the symbiotic algæ before hatching. This view is supported by the fact that all the infected polyps of the Woods Hole region were found in a comparatively restricted locality.

## Summary.

The feeding reactions of *Astrangia* in general consist of four actions: muscular action of the tentacles, muscular action of the central part of the oral disk, secretion of mucus by the oral disk, and ciliary action of the stomodæum.

The reaction of the food-vacuoles in the digestive region of the mesenterial filaments immediately after the feeding is acid; after about two days the reaction changes to alkaline. In this alkaline

period in all probability the digestion takes place. There is sufficient evidence that besides intracellular digestion a secretion of a digestive fluid (a trypsin-like enzyme) occurs.

In the polyps of *Astrangia* which contain zoöxanthellæ in their entoderm these unicellular algæ furnish a part of the normal food of the polyps: a quantity of these algæ are digested in the mesenterial filaments.

Polyps of *Astrangia* without zoöxanthellæ can be easily infected with these algæ by feeding them with crab meat mixed with parts of the tissues of strongly infected polyps.

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# A STUDY OF OXYGEN METABOLISM IN *DROSOPHILA MELANOGASTER.*

M. R. CLARE.

## Introduction.

Although *Drosophila melanogaster* is uniquely favorable as material for genetical studies, relatively few physiological investigations have been conducted upon this animal. Its small size has certainly been a deterrent to such studies; yet, with suitable apparatus, this feature is unimportant. To supplement the remarkably full record we possess for its genetical behavior physiological studies are especially desirable, and an attempt is made in this paper to show the practicability of investigations into the metabolism of this fly and also to illustrate the type of results which such study can be expected to yield. The investigation was undertaken primarily to determine to what extent degree of inbreeding may be reflected in metabolism.

Measurements were made of the oxygen consumption of *Drosophila* pupæ, and these proved admirably adapted to this purpose. At no time during pupal life except at its extreme termination are results complicated by muscular movements, hence standard metabolism alone is measured. Moreover, the fly is so amenable to conditions of laboratory culture that experimental pupæ are available at all times and can be grown under standard conditions.

The investigation was conducted at the University of Pennsylvania, for which privilege the writer desires to acknowledge his indebtedness to Doctor C. E. McClung. He is under special obligations to Doctor J. H. Bodine, who not only suggested the problem but was ever ready with helpful suggestions throughout the progress of the work. The stocks of experimental flies were kindly contributed by Doctors C. B. Bridges, H. J. Muller, L. E. Griffin, Chas. Zeleny, J. H. Bodine and R. L. King.

## Material and Methods.

Eight stocks of "wild" *Drosophila melanogaster* were employed in the study. Three of these were caught shortly before the

work was undertaken in localities removed from centers where flies are grown. These may be called the "non-inbreds." The remaining five stocks had been inbred for a number of years before being received and, as some of these are well known, the following table of sources may be of interest.

TABLE I.

SHOWING DERIVATION OF EXPERIMENTAL MATERIALS.

NON-INBREDS.

| Designation. | Source. | Captured. |
|---|---|---|
| G........... | Portland, Oregon | August, 1923 |
| H........... | Hellam, Penna. | July, 1923 |
| B........... | Rockaway, N. J. | July, 1923 |

INBREDS.

| Designation. | Original Name. | Source. | Inbred Since |
|---|---|---|---|
| C–2.......... | "Ossining" | Columbia University | 1921 |
| C–1.......... | "Pt. Pleasant" | Columbia University | 1921 |
| F........... | "Florida No. 5" | University of Texas | 1918 |
| I........... | —— | University of Illinois | 1916 |
| P........... | | University of Penna. | Many years |

Owing to some preliminary difficulties, work was not begun until December, 1923, or until the non-inbred stocks had become inbred for several generations. Thereafter readings were continued, with interruptions, until December, 1924, when the experimental work was concluded. The chronological distribution of the work is without significance to our study and will not be entered into.

The pupæ of *Drosophila melanogaster* are so small that it is impracticable to make an extensive series of measurements of oxygen consumption on single pupæ, hence lots of 10 or fewer pupæ were used for each determination. Readings were taken over a period of 4 or 5 hours each day throughout the duration of pupal life. Data were collected for 160 lots of pupæ.

The stock flies were grown in mass culture in large quinine bottles and kept at room temperature. The experimental pupæ, however, were always the products of single matings. From time to time the flies in a culture bottle were removed and matings were made up from new flies as they appeared which

were never more than 20 hours old. The pairs of flies were cultured in shell vials (about 9 cm. long by 2 cm. diameter) containing banana agar and were transferred each day to fresh vials so that the pupæ forming in a particular vial resulted from eggs deposited therein on a single and known day. A complete cultural record was kept which included figures for the sex ratios of the flies appearing in all of the vials in order that a check might be had on any conditions of metabolism attributable to sex peculiarities of the matings. It happened, however, that for all of the matings the distribution of the sexes remained normal. Some of the matings were cultured at variable room temperature, ranging from 21° C. to 25° C. about a mean of 23° C.; others were cultured in an incubator at a constant temperature of 25° C. It is necessary to stress this distinction, for upon it will be based a natural division of the data into two parts. Hereafter, the pupæ formed at room temperature will be referred to as of the "first experimental period," whereas those formed at 25° C. will be referred to as of the "second experimental period."

The banana-agar was prepared according to the usual method and while still liquid about 5 or 6 cc. of the material were introduced into each previously sterilized vial, which was provided with a cotton plug. Usually a sufficient number of vials was prepared at a time to supply requirements for two or three days and kept in a refrigerator while awaiting use. Before introducing a pair of flies into a fresh vial, a small amount of powdered Magic Yeast was dusted on the surface of the culture medium and on this was placed a disc of towel paper cut somewhat smaller than the bore of the vial. When a very limited amount of paper is placed in a vial, the larvæ developing therein pupate on the glass without "spinning" and therefore require a minimum of cleaning in preparation for use. They can then be removed quite readily from the glass without danger of injury with a small brush after a preliminary wetting with water.

Whenever possible, the first 10 pupæ appearing in a vial were used for a determination, but quite frequently only a smaller number could be secured. Each evening the vials for the several matings were examined and any pupa which had appeared unduly early was checked with a wax pencil in order that it

might not be included among the experimental pupæ selected the following morning. Accordingly, the maximum age of pupæ on which determinations were made was 15 or 16 hours, a point which must be kept in mind.

In preparation for the first reading a lot of pupæ was first washed in water with a camels' hair brush, then treated with 80 per cent. alcohol for 2 or 3 minutes to destroy any adhering yeast cells, rinsed in water and dried on filter paper. After being weighed on a delicate balance they were placed in a cotton-lined basket and suspended in the oxygen-measuring apparatus. Between determinations each lot of pupæ was kept in an individual moist chamber with a piece of moist filter paper. Weighings as well as determinations were continued each day until development was so far advanced that there was danger of flies emerging. As a final step, record was made of the sexes of the flies which issued from each lot of pupæ.

Rates of oxygen consumption were measured with an improved form of the manometer of Krogh (1915), described by Bodine and Orr (1925). Six manometers were used, a single one only being used for a given lot of pupæ during the period of pupal life. During readings the manometers were placed in a water bath which was kept at a constant temperature. From day to day, however, the temperature of the bath varied in accord with the temperature of the room, but this fluctuation is not registered in the determinations as oxygen values are always reduced to 0° C. Calculation of rates has been made on the basis of oxygen consumption per minute of time and both per gram body weight and per single pupa.

## The "Oxygen Curve."

The duration of pupal life is influenced largely by temperature. An index of this correspondence is afforded by figures for the number of days on which oxygen determinations were possible—even though such figures do not represent the actual duration of pupal life. During the first experimental period 4-day pupæ were predominant, 5-day pupæ occurring rarely and 3-day pupæ only to the extent of 7 per cent. At a temperature of 25° C., on the other hand, the percentage of 3-day pupæ was increased to 60 per cent., while 5-day pupæ did not occur.

During the course of pupal development certain readjustments are in progress which are reflected in rates of metabolism. In Fig. 1 the changes in metabolism are shown in the form of curves. Those for 4-day pupæ are derived from a single mating of the second experimental period while those for 5-day pupæ pertain to the first period. Each curve shows an initial fall after the first day of pupal life succeeded by an abrupt or gradual rise. The most instructive are the curves for 5-day pupæ in which the period of depression of metabolic rate is seen to continue from the second to the third day. The curves for 4-day pupæ are obviously abbreviations of these.

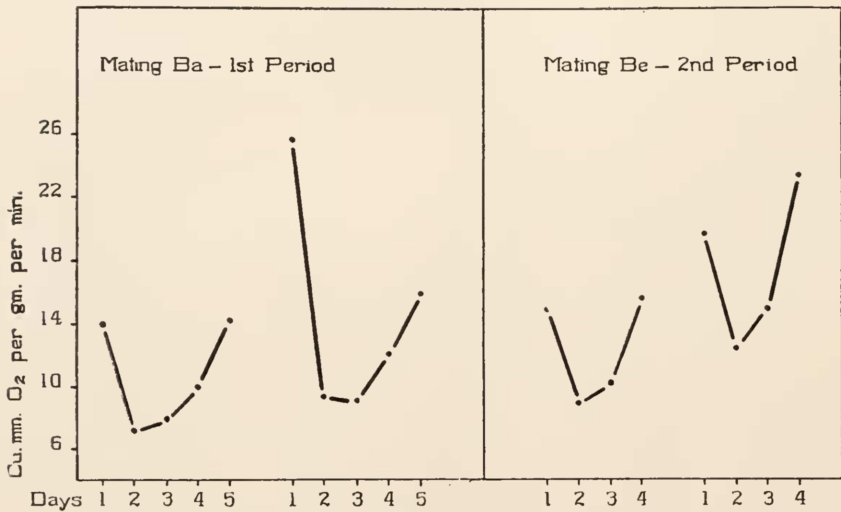


Fig. 1. Metabolism curves based on $O_2$ consumption per minute per gram of body weight. Ordinates = $O_2$ values in cubic millimeters; abscissæ = time in days.

This type of curve appears to be characteristic of pupæ in general, the modifications presented in the several species that have been investigated being due chiefly to the varying extensions of the period of depression. The significance of this type of pupal curve has been discussed at length by several workers among whom may be mentioned Tangl (1909, 1 and 2), Weinland (1906), and quite recently Fink (1925). The researches of Weismann, Perez, and others have demonstrated that early in pupal life the persisting larval tissues undergo a series of histolyses

leading to their ultimate dissolution, and that the tissues of the imago are built up through the activity of certain groups of cells which survive histolysis and appear to be set aside for this specific purpose. In other words, two distinct processes are in progress during pupal life—a destructive and a constructive, the latter being inaugurated before the completion of the former. The authors cited identify the abrupt fall in metabolism early in pupal life with the histolytic process and the recovery after the period of depression with the formation and growth of imaginal tissues.

## Variability in Rates of Metabolism.

When a series of curves for rates of oxygen consumption is examined, a feature which is most striking is the considerable variability in values exhibited. The majority of the values for any one day of pupal life fall within fairly narrow limits, but scattered among these are numerous others representing very high as well as rather low rates, distributed in a seemingly erratic manner. An early examination of the data proved that most of the very high rates belonged to lots of pupæ, one or more members of which not only failed to give issue to flies but failed to pass beyond the stage of development characteristic of second day pupæ. Instances of this sort suggest that probably intestinal microörganisms find their capacity for growth released by the death of the pupa or pupæ harboring them, and by their rapid multiplication elevate the rate of oxygen consumption to a high level. With one exception to be considered later, it was deemed necessary to completely eliminate from further consideration all data pertaining to lots of pupæ showing incomplete development, thereby reducing the total number of determinations by about one third. Several further eliminations are for lots of pupæ which were accidentally shaken from their supports within the manometers into the 2 per cent. NaOH solution over which they were suspended. Although washed as quickly as possible in a large volume of water and again set up in the manometer, a lot so treated almost invariably responded with an abnormal elevation of rate. Mention should also be made of a series of determinations which was set aside because of bacterial contamination

of the cultures. This condition resulted in the production of a limited number of very small pupæ of exceptionally low weights. A single example from this series has been retained in Table 5 in order to show that, in spite of the very low pupal weights, the rate of metabolism is to all appearances normal. The rather drastic elimination which has been practiced has reduced to a considerable degree both the number of determinations and the variability in rates, but that the latter has been by no means removed is shown in Fig. 2.

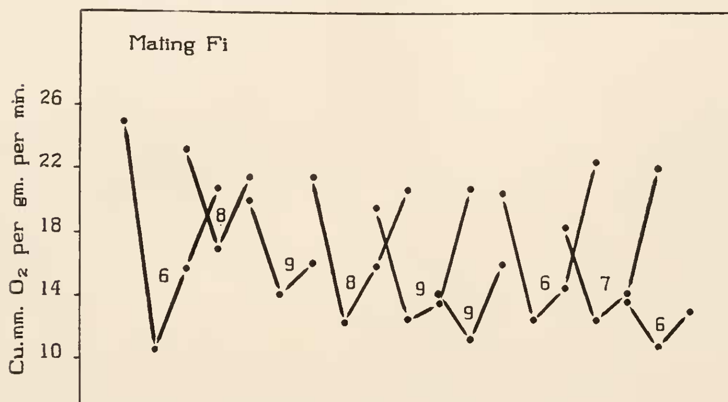


FIG. 2. Metabolism curves showing variability in rates within a single mating, cultured at 25° C. Numbers indicate particular manometers used in establishing rates. Ordinates = $O_2$ per gram per minute, in cu. mm.

There are several conceivable factors which might underlie this variability and they will be taken up in turn. It is obvious that if comparisons are to be made between the several stocks in respect to oxygen consumption this irregularity must be reduced to a minimum. Suspicion was at once cast on the calibration of the manometers used in making the determinations. That these are not the primary source of the variability will be evident from a further consideration of Fig. 2. The curves of this figure are for lots of pupæ all of which are offspring of a single pair of flies. Associated with each curve is a number which is that of the manometer used in determining the rates. For each of two manometers, numbers 6 and 9, three sets of determinations are represented which, it should be noted, exhibit marked variability in rates of metabolism. The manometers were very carefully

calibrated several times and any possible errors from this source, although carefully looked for, have eluded detection.

## Pupal Weights and Metabolism.

In studies on respiratory metabolism the practise has been to emphasize the relation between weights of experimental organisms and intake of oxygen or production of carbon dioxide, on the assumption that for comparable samples of living material within a species, the rates of metabolism remain fairly constant. Rubner, along with others, on the other hand, has instituted the procedure of basing metabolism on extent of body surface, arguing, in the words of Krogh (1916) that "the metabolism is simply a function of the conditions for loss of heat, while there is no such thing as a specific oxidative activity of the cell." Rubner later saw fit, however, to qualify this idea. These practises were derived from investigations on warm-blooded animals possessed of a heat-regulating mechanism, and their applicability to invertebrate animals is extremely uncertain. Unfortunately, the studies on metabolism in invertebrates have been so few in number and have afforded results of so conflicting a character that conclusions based on them do not seem to be justified. We feel obliged, therefore, to examine the data for *Drosophila* with some care and determine, if possible, the significance to be attached to weight; and attempt to decide whether or not rates of metabolism are subject to decided change. No data are available on which to base figures for pupal surface.

Pupal weights exhibit a wide range in value, the means for newly-formed or first-day pupæ in lots of 10 varying between 11 and 15 milligrams. At the extremes of the range lots of 10 pupæ may possess a weight as low as 9 mg. or as high as 17 mg. For the products of a single mating, likewise, the range of variability is very pronounced.

In addition to the rôles played by food and overcrowding as factors affecting pupal weight—and these can hardly be considered as applying in this work—temperature certainly is a determining agency. An examination of Table 2 shows that pupal weights of the second period are significantly lower than those for the first period. In the summer of 1923, during a

## Table II.

Pupal Weights and Oxygen per Gram for Total Stocks.

Figures for weights are based on lots of 10 pupæ, stated in milligrams; figures for oxygen consumption are rates per minute, stated in cubic millimeters.

| | 1st Day. | 2d Day. | 3d Day. | 4th Day. |
|---|---|---|---|---|
| First Period—4-Day Pupæ. | | | | |
| Weights | 14.04 ± .20 | 12.57 ± .17 | 12.46 ± .17 | 12.36 ± .18 |
| $O_2$ per Gram | 15.89 ± .34 | 9.00 ± .15 | 10.38 ± .21 | 15.44 ± .42 |
| Second Period—4-Day Pupæ. | | | | |
| Weights | 13.30 ± .18 | 11.87 ± .15 | 11.78 ± .17 | 11.65 ± .18 |
| $O_2$ per Gram | 18.35 ± .44 | 11.00 ± .20 | 13.30 ± .35 | 21.15 ± .35 |
| Second Period—3-Day Pupæ. | | | | |
| Weights | 13.45 ± .18 | 12.25 ± .14 | 12.06 ± .14 | |
| $O_2$ per Gram | 17.94 ± .38 | 12.28 ± .28 | 16.71 ± .36 | |

period when the room temperature varied between 25° and 30° C., a collection of weights was made which for lots of 10 pupæ present mean values of 11.83 ± .152, 10.86 ± .169 and 11.00 ± .194 for the three days of pupal life. It is, therefore, evident that an inverse relation obtains between temperature and pupal weight. When the range of temperature is not very great,

## Table III.

Coefficients of Variation for Pupal Weights and Oxygen per Gram.

The coefficients are based on the figures presented in Table II.

| | 1st Day. | 2d Day. | 3d Day. | 4th Day. |
|---|---|---|---|---|
| First Period—4-Day Pupæ. | | | | |
| Weights | 11.2 ± 1.03 | 10.4 ± .97 | 10.4 ± .97 | 10.7 ± 1.02 |
| $O_2$ per Gram | 16.5 ± 1.51 | 13.0 ± 1.22 | 15.1 ± 1.41 | 20.3 ± 1.93 |
| Second Period—4-Day Pupæ. | | | | |
| Weights | 9.8 ± .97 | 9.1 ± .90 | 10.3 ± 1.02 | 10.2 ± 1.09 |
| $O_2$ per Gram | 17.3 ± 1.72 | 13.1 ± 1.30 | 18.5 ± 1.84 | 10.9 ± 1.16 |
| Second Period—3-Day Pupæ. | | | | |
| Weights | 11.3 ± .94 | 9.9 ± .83 | 9.4 ± .80 | |
| $O_2$ per Gram | 18.3 ± 1.52 | 19.2 ± 1.62 | 17.9 ± 1.53 | |

however, this correlation does not manifest itself when curves for temperature and pupal weights are compared. Moreover, a constant temperature serves merely to limit somewhat the range of fluctuation, instead of insuring stability, as can be seen from Table III. Temperature, then, operates only in a large way in its influence on weight of pupæ.

Corresponding to the sudden drop in metabolism shortly after the inauguration of pupal life, there occurs a marked drop in pupal weight. Sometimes this loss is relatively enormous, in other exceptional cases relatively insignificant. Unlike metabolism, however, there is no recovery in weight after the second day, the level for the third and fourth days remaining approximately identical with that for the second day. Sometimes for these latter days a slight increase is registered but as frequently a slight fall occurs.

The interesting sequence of changes during the pupal period in respect to pupal weights and metabolism is presented in Fig. 3. The data on which this figure is based represent a mating selected for illustration because a large number of determinations is available and the pupal weights show an unusual consistency of level. The figure is divided into parts corresponding to the several days of pupal life and a common scale is used which enables one to carry out comparisons between any two days.

The relationship between the curves for metabolism per pupa and per unit body weight affords an index of the ratio existing between weight of respiring tissue and that of inert materials associated with the respiring tissue. When the two curves follow a strictly parallel course this ratio must remain constant in the several lots of pupæ, provided complicating factors are not present. When for a particular determination the curves become approximated, the inference may be made that the relative amount of non-respiring or inert materials is reduced, whereas, when they become more widely separated, this condition may be attributed to a relative increase in the non-respiring materials present. It will be clear, therefore, that in general the ratio of respiring to non-respiring substances in the pupæ on which Fig. 3 is established remains a fixed quantity. Interesting exceptions, however, appear in the first and last determinations, numbers 8

and 37. In the latter the relation of the metabolism curves for the first day indicates that the excessive weight of the pupæ in question was due to the presence of a disproportionately great amount of non-respiring material, let us suggest water. As pupal development proceeded, this disproportion gradually became

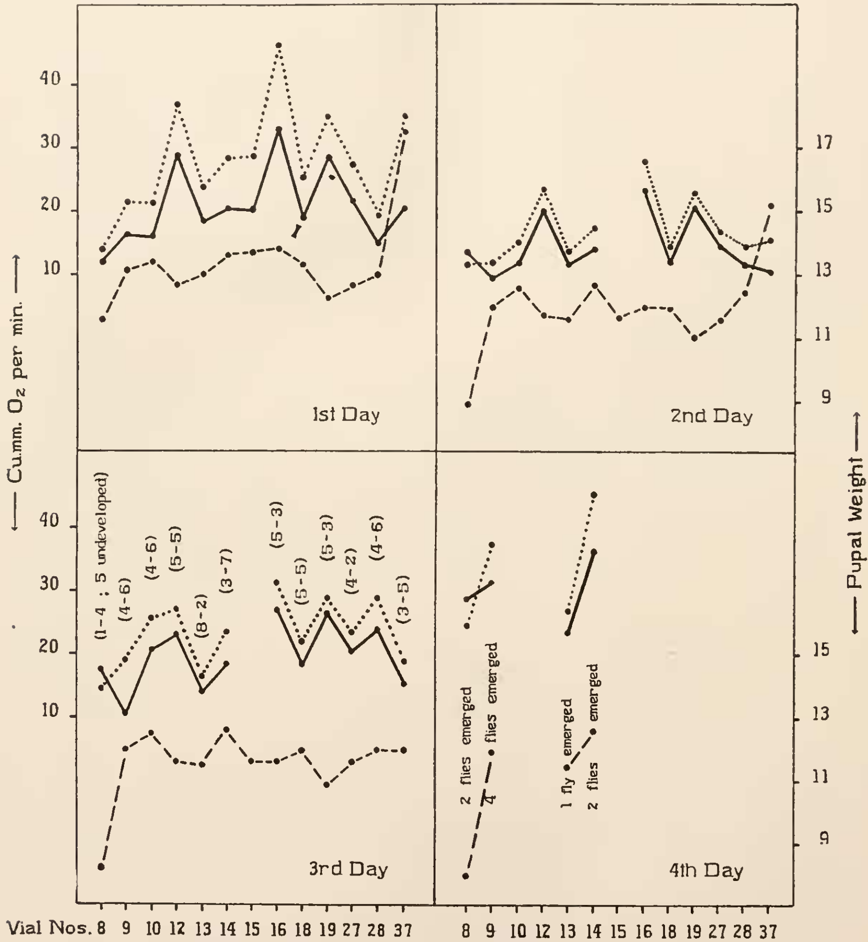


FIG. 3. Modifications in metabolic rates and pupal weights during pupal life for lots of pupæ derived from a single mating, C–2:4, at 25° C. Corresponding values for each day of pupal life are connected into curves. Upper curve = $O_2$ per pupa; middle curve = $O_2$ per gram and lower curve = pupal weights. Ordinates at left are $O_2$ values in cu. mm., at right are pupal weights in milligrams per 10 pupæ. Abscissæ are numbers of the vials from which experimental pupæ were obtained. Sex-ratios in brackets (males—females).

reduced until, on the third day, the normal balance of respiring and non-respiring materials was restored. The first determination, number 8, on the other hand, presents a different story which is complicated by the fact that 5 of the 10 pupæ on which this determination is based failed to complete their development, the only case of the sort we shall consider. The approximation of the metabolism curves suggests that the pupæ contained a very small amount of non-respiring material relatively to the respiring tissues, yet this idea does not seem to harmonize with the remarkable fall in pupal weight occurring after the first day of pupal life. If, however, we postulate for this case, as was done earlier for cases of arrested development in general, a bacterial activity which would serve to elevate the metabolism per unit body weight relatively to the total metabolism, we develop an interpretation which affords an understanding not only of the peculiar relation shown by the two curves but also of the cause for the unusual decrease in pupal weight. Instances of this sort, representing a fluctuation in the relative amounts of respiring and non-respiring substances present in pupæ, are of frequent occurrence and are further illustrated in Fig. 5. The only general statement that seems permissible is that pupæ of very low weight usually show an elevation of the rate per unit weight relatively to the total metabolism but this condition is by no means invariably true. It appears, therefore, that mere weight is a rather unreliable index of the amount of respiring tissue.

It is manifest that for the first and second days of pupal life a rough proportionality certainly exists between pupal weights and metabolism, thus explaining in a way the trends of the oxygen curves. However, the peculiarities of the 12th, 16th and 19th determinations must receive an interpretation of their own. As the two metabolism curves remain closely parallel, the only conclusion that seems justifiable is that the rates for the determinations in question are of a discontinuous type; in other words, independent of pupal weight. It would appear, therefore, that strictly comparable samples of respiring tissue grown under, and subjected to, like environmental conditions can nevertheless exhibit marked diversity in rates of metabolism. It is ex-

ceedingly improbable that the independent rates of these determinations are due to a secondary source, bacterial for example, contributing in an additive manner to the respiring pupal tissues. If this were the case, we should expect the condition to be indicated by the metabolism curves.

As applied to *Drosophila*, the practice of bringing metabolism into relation with pupal weight is warranted only in a most general way. The correlation is most pronounced during the first day of pupal life when the coefficient of correlation for 4-day pupæ grown at 25° C. attains a value of $0.47 \pm .071$. On the second and subsequent days this value is considerably reduced. Too much importance should not be attached even to this correlation. The relationship often fails completely in individual cases, as is shown in Fig. 5. This fact, taken in connection with the rather frequent tendency toward the establishment of discontinuous rates, indicates that an understanding of metabolism in *Drosophila* is not to be sought on the basis of weight of respiring tissue but rather through the impress of factors regarding whose nature we are at present in ignorance.

A point of considerable interest brought out in Fig. 3 and in other figures for similarly prepared material is the constancy of the relation between the respiratory rates for the individual lots of pupæ up to the third day of pupal life. The striking similarity of the metabolism curves for the first and second days of pupal life indicates that the rates for this period are relatively stable and that the establishment of independent rates may occur either before or later but hardly during this period. On the third day, however, after the inauguration of growth and differentiation of imaginal tissues, a new order of rates is ushered in, which is that of adult life. As the metabolism curves for the third day in Fig. 3 show, these new rates considered in their entirety still preserve to a slight degree their kinship with those of early pupal life, but each lot of pupæ now develops along a new course. It appears that the rates of first-day pupæ in no way can serve to forecast those of the flies which will later emerge from them. We have already suggested that the period of institution of new adult rates is a somewhat critical stage in pupal development. The great majority of pupæ of arrested

development, apart from those containing fully formed flies unable to escape from the pupal cases, represented the stage of development characteristic of 2-day-old pupæ. In other words, the histolytic process had been gone through but the reconstructive had failed of attainment.

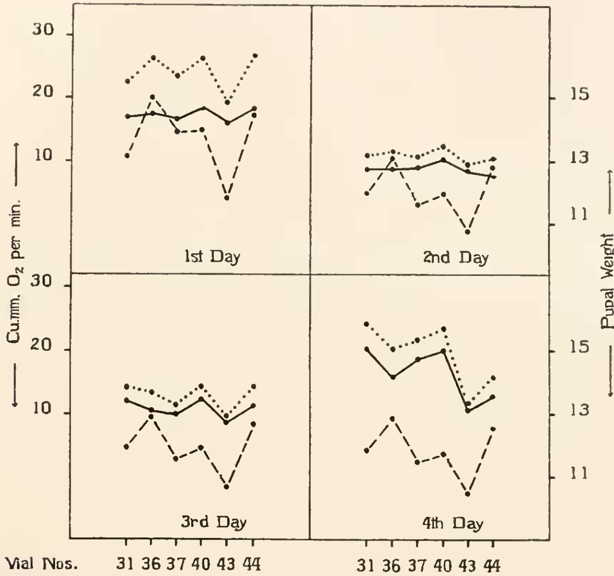


FIG. 4. Metabolic rates and pupal weights for pupæ of the first experimental period, products of single mating Hd. Upper curve = $O_2$ per pupa; middle curve = $O_2$ per gram; lower curve = pupal weights. Ordinates = $O_2$ values in cu. mm. at left, pupal weights in mg. per 10 pupæ at right. Abscissæ = numbers of the vials from which experimental pupæ were obtained.

Do flies about to emerge possess a higher standard metabolism than do larvæ? Our selected figure throws some light on this question. The elevated rates for the four-day pupæ in the figure are certainly in large part to be explained as due to muscular contractions incident to emergence of the flies, and accordingly these records do not indicate standard metabolism. When we recall that the figures for the first day of pupal existence are for pupæ which may be as old as 15 or 16 hours and in which histolysis and its accompanying depression in rate of metabolism has perhaps been in progress for an unknown length of time, it would appear from a comparison of the rates for the first and

third days of pupal development that the standard metabolism of larvæ is actually somewhat greater than that of the flies although, relatively to body weight, the two stages may be more nearly comparable.

Before turning from the general subject we have been considering, a point of difference between the metabolism curves for the first and second experimental periods should be noted. For a cultural temperature of 25° C., we have seen that marked fluctuations occur in rates of oxygen consumption among lots of pupæ derived from a single mating, and corresponding in a very rough way with variations in pupal weight. In material representing the first experimental period at a lower temperature, the extent of fluctuations in metabolism is less pronounced until the last day when it becomes increased. Pupal weights of the first period are more irregular than for the second period and in consequence the parallelism between weight and metabolism tends to become lost. The relationship between pupal weight and metabolism for this period is represented in Fig. 4.

## Influence of Sex on Metabolism.

It has been pointed out that for any one day of pupal life the metabolic rates for different samples of pupæ vary considerably and that weight is a very untrustworthy guide in arriving at an understanding of this irregularity. Is sex a contributing factor? Biological literature abounds in references to the physiological distinctness of the sexes and several important recent researches have depended for interpretation on the postulate that male animals possess a higher rate of metabolism than do female. So far as the writer knows, only one experimental attempt has been made to measure this supposed difference directly, namely, the investigation by Benedict and Emmes (1915). With human subjects these workers found a slight increase in metabolism in favor of males over the rates for females.

There are certain facts at hand to suggest a possible difference in metabolism between the sexes of *Drosophila*. At times, at least, there is a well developed tendency for female flies to emerge earlier than the males from the first-formed pupæ. The present data happen to be inconclusive on this point. The

## Table IV.

### Sex and Pupal Weights.

Pupal weights are stated in milligrams for lots of 10 pupæ.

*First Day.*

| ♂♂ | Pupal Weights. | | | | | | | | | | ♀♀ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | |
| 1 | | | | | | | | | | | 9 |
| 2 | | | | | | | × | 14.30 ± 1.187 | | | 8 |
| 3 | | | | | | | × | 14.37 ± 0.780 | | | 7 |
| 4 | | | | | | × | | 13.35 ± 1.630 | | | 6 |
| 5 | | | | | | × | | 13.90 ± 1.337 | | | 5 |
| 6 | | | | | × | | | 12.87 ± 2.152 | | | 4 |
| 7 | | | | | × | | | 12.79 ± 1.778 | | | 3 |
| | | | | | | | | | | | 2 |
| 9 | | | | | | | | | | | 1 |

*Second Day.*

| | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | 9 |
| 2 | | | | | | × | | 12.90 ± 1.300 | | | 8 |
| 3 | | | | | | × | | 13.00 ± 0.223 | | | 7 |
| 4 | | | | | × | | | 12.08 ± 1.245 | | | 6 |
| 5 | | | | | × | | | 12.38 ± 1.132 | | | 5 |
| 6 | | | | × | | | | 11.64 ± 1.679 | | | 4 |
| 7 | | | | × | | | | 11.58 ± 1.418 | | | 3 |
| 8 | | | | | | | | | | | 2 |
| 9 | | | | | | | | | | | 1 |

*Third Day.*

| | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | 9 |
| 2 | | | | | | | | | | | 8 |
| 3 | | | | | × | | | 12.66 ± 0.145 | | | 7 |
| 4 | | | | | × | | | 12.00 ± 0.107 | | | 6 |
| 5 | | | | | × | | | 12.31 ± 0.175 | | | 5 |
| 6 | | | | × | | | | 11.54 ± 0.278 | | | 4 |
| 7 | | | | × | | | | 11.72 ± 0.213 | | | 3 |
| 8 | | | | | | | | | | | 2 |
| 9 | | | | | | | | | | | 1 |

records do, however, suggest a tendency for larvæ destined to become females to pupate earlier than those which will develop into males. Also, as is shown in Table IV., pupæ which are to become females incline toward heavier weight than those which become males.

The sex ratios for a typical series of determinations have been indicated in Fig. 5 as well as in Fig. 3. All of the data have

been gone over most carefully with the unqualified result that sexual differences in metabolism are impossible to detect. If such exist they must be so small that they are obscured by irregularities induced by other agencies.

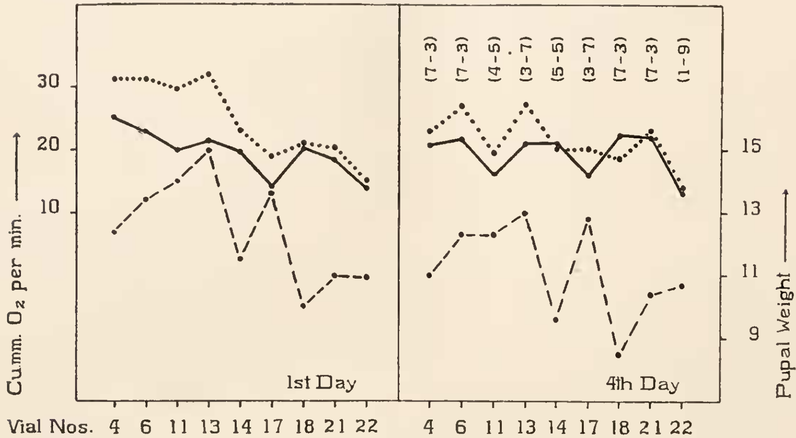


FIG. 5. Oxygen rates, pupal weights and sex-ratios for lots of pupæ of mating Fi, of the second experimental period. Curves for the second and third days of pupal life are omitted. Sex-ratios shown in brackets, the first figure for males, the second for females. Upper curve = $O_2$ per pupa; middle curve = $O_2$ per gram; lower curve = pupal weights. Ordinates at left are $O_2$ values in cu. mm., at right are pupal weight values in mg. per 10 pupæ. Abscissæ refer to the vials from which experimental pupæ were obtained.

## Further Considerations on Irregularity in Respiratory Rates.

The most significant result which this study has revealed is the peculiar irregularity in rates of metabolism exhibited especially by pupæ formed under conditions of constant temperature. It might be remarked that there is no ground for believing that the causes underlying this irregularity are genetic in character. On the contrary, the usual tendency of the rates for lots of pupæ derived on consecutive days from a common source to exhibit a graduated character either in the direction of elevation or of depression strongly suggests the effect of graduated environmental influences. We have already eliminated pupal weight and sex as important agencies responsible for irregularity in rates and we may now refer to several remaining possibilities.

It is believed that under the conditions of the experiment food conditions were maintained as nearly uniform as is possible. Moulds occasionally became established in the culture vials but the growth rarely if ever became noticeable until after the larvæ had pupated. It is altogether possible that organisms harmful to the larvæ and of sporadic occurrence in the tubes may have contributed in slight measure to the irregularity, but this is hardly a likelihood. We have referred to the bacterial contamination of one series of cultures, with the consequent reduction in weight of the pupæ. Curiously enough, this reduction in weight does not appear to have induced a parallel modification in rate of metabolism.

The pupæ of *Drosophila*, it would seem, are adapted to resist conditions of desiccation, at any rate for short periods. On several occasions lots of pupæ were inadvertently subjected overnight to the drying effect of the room air. This treatment produced no detectable effect on the following day either in pupal weight or in rate of oxygen consumption. This point is of some interest in connection with the results obtained by Caldwell (1925), who has found that the metabolism of some animals, as measured by carbon dioxide production, is modified as a result of desiccation.

A degree of irregularity in oxygen rates was certainly introduced with varying ages of the pupæ used. Larvæ developing from eggs deposited on a single day possess individual peculiarities, some pupating precociously, others delaying the act for a day or longer after their brothers and sisters have made the change. Accordingly, all of the pupæ thrown together for a determination are not of the same age. Among lots of experimental pupæ, however, the mean ages should be nearly identical as selection was performed at about the same hour each morning and the manometer readings were distributed over approximately identical time periods each day.

Mention might be made of an attempt to find an interpretation of the irregularity in rates through a study of varying intensities of such environmental factors as light, humidity, etc., data for which were secured from the local weather office. As might be expected, this attempt was not fruitful of results. Likewise, a

careful examination of data on length of larval life, of pupal life, etc., proved barren of results, except that a suggestive but very rough correspondence between rates of oxygen consumption and productivity, as measured by the number of flies appearing in the vials, did come to light. A correspondence of this sort would carry the implication that the metabolic rates of the pupæ are due to an impress set by the metabolic condition of the female parent at or about the particular time the eggs are deposited, and that this impress continues without impairment over the period of larval life. The data at hand do little more than suggest this possibility.

## Effects of Inbreeding.

The central question around which this study was planned was whether or not it is possible to find an index of inbreeding in rates of metabolism. If metabolism were subject to control as a result of genetic make-up, we should expect to find evidence of it reflected in our results. There is not only no indication that heredity plays any but the most general rôle in metabolism; but, on the contrary, we have seen some reason for believing that rates are at the command of graduated environmental influences. The irregularity in rates which we have stressed renders it impossible to make satisfactory comparisons between different matings within a common stock, and the propriety of lumping all of the matings of a stock under a mean is very questionable. However, this has been done in Table 5 in which are presented means for most of the stocks. The eliminations previously referred to made such serious inroads on the data for the majority of the matings that Table V. must be built up on an inadequate number of determinations. The figures for stock G of the first period are based on as few as 3 determinations; and in all other cases on 4 determinations except where a probable error is attached, this indicating that 5 or more determinations were available. Deficient as it is, this table illustrates the impossibility of separating inbred from non-inbred stocks on the basis of rates of metabolism.

## Summary.

Several results emerge from this study which, it is believed, should assist in defining the problem and, at the same time,

## Table V.

Pupal Weights and Oxygen per Gram (in Cu. Mm. per Minute) for Individual Stocks.
Non-inbred stocks are starred (*).

| Stocks. | 1st Day. | | 2d Day. | | 3d Day. | | 4th Day. | |
|---|---|---|---|---|---|---|---|---|
| | Weight. | $O_2$ per G. | Weight. | $O_2$ per G. | Weight. | $O_2$ per G. | Weight. | $O_2$ per G. |
| **First Period—4-Day Pupæ.** | | | | | | | | |
| H* | 14.25 ± .31 | 17.12 ± .42 | 12.62 ± .16 | 9.50 ± .18 | 12.62 ± .16 | 10.62 ± .29 | 12.62 ± .16 | 15.75 ± .73 |
| G* | 13.00 | 11.50 | 12.50 | 8.50 | 12.50 | 11.00 | 12.50 | 15.50 |
| P | 15.40 ± .15 | 15.20 ± .48 | 13.80 ± .12 | 8.60 ± .31 | 13.40 ± .24 | 10.20 ± .22 | 13.25 | 15.70 |
| I | 14.33 | 15.25 | 12.66 | 8.00 | 12.66 | 9.00 | 12.33 | 12.66 |
| C-2 | 12.20 ± .35 | 16.40 ± .84 | 10.50 | 9.50 | 10.25 | 10.70 | 10.25 | 15.20 |
| C-1 | 14.66 | 17.33 | 13.00 | 9.66 | 13.00 | 11.00 | 13.00 | 17.33 |
| **Second Period—4-Day Pupæ.** | | | | | | | | |
| H*[1] | 11.71 | 17.42 | 10.71 | 9.71 | 10.40 | 11.75 | 10.66 | 17.00 |
| G* | 13.75 | 17.50 | 12.50 | 10.00 | 12.25 | 12.50 | 12.25 | 20.50 |
| I | 14.25 | 14.75 | 12.25 | 9.25 | 12.25 | 11.50 | 12.25 | 18.25 |
| C-2 | 13.60 ± .15 | 19.60 ± .67 | 12.20 ± .12 | 12.00 ± .38 | 12.20 ± .12 | 14.00 ± .63 | 12.00 | 23.50 |
| F | 12.60 ± .33 | 19.30 ± .74 | 11.30 ± .28 | 11.60 ± .24 | 11.20 ± .34 | 14.60 ± .46 | 11.10 ± .31 | 22.10 ± .29 |

[1] The low weights of the pupæ of stock H of the second period were due to pollution of the cultures by bacteria.

should suggest types of investigation which are most likely to contribute to an understanding of metabolism in *Drosophila melanogaster*.

It has been shown that a close approach to a knowledge of metabolism in *Drosophila* pupæ cannot be made if sole dependence is placed on weight of respiring tissues as a guide. In a very rough way, pupal weights show a correspondence with the trends of metabolic rates, but, for particular matings, especially when the larvæ are maintained at lower temperatures, the correspondence is largely lost. It has also been shown that rates of oxygen consumption are very irregular and vary in a manner highly suggestive of the influence of environmental agencies. The not infrequently pronounced elevations above the general level of metabolism lend support to the conclusion that the metabolic rates for comparable samples of respiring tissue are not necessarily fixed within narrow limits. Finally, it has proved impossible to find a metabolic difference between the sexes or to detect any difference in metabolism between inbred and non-inbred stocks.

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# THE ACTION OF ETHER ON PROTOPLASM.[1]

L. V. HEILBRUNN,

No problem in general physiology has been investigated more frequently than the problem of anesthesia. Many workers have attempted to discover the essential nature of the action of anesthetics on living substance. Not only have animals, tissues, and cells been studied, but some physiologists have even gone so far as to study the effect of anesthetics on lifeless materials which they believed similar to protoplasm.

Many theories have been proposed to explain anesthesia. In recent years the permeability theory has had a wide following. This theory claims that anesthetics either decrease the permeability of the cell, that is to say of the plasma membrane, or that they at any rate prevent an increased permeability. The actual evidence in support of the theory is somewhat scanty. What there is has been well summarized by Winterstein (1), Höber (2), and Lillie (3), all three of whom are strong advocates of the theory.

In only a few cases has it been shown that anesthetics lower cell permeability and most of these cases are doubtful, as Winterstein admits. In some instances observers have claimed that anesthetics produce an increase rather than a decrease in permeability (see Höber). Perhaps the work most often quoted in support of the doctrine that anesthetics lower permeability is the series of conductivity measurements of Osterhout (4) on plant cells. There is however a growing realization that a decrease in the electric conductivity measurements of a group of cells does not necessarily depend on a decrease in the permeability of the plasma membranes of these cells. Many factors play a part in influencing the conductivity measurement. When an electric current is sent through a mass of cells, certainly a large part of the current goes between and around the cells. Changes in the conductivity of the spaces between cells are therefore apt

[1] Contribution from the Zoölogical Laboratory of the University of Michigan.

to be of greater moment than changes in the conductivity of the cells themselves. The factors involved include possible changes in the size and shape of the spaces between cells, as well as changes in their conductivity. One such factor is perhaps of especial importance for substances like the ordinary fat-solvent anesthetics which alter the viscosity of the medium in which they are dissolved. This is the effect of a change in the viscosity of a solution on its conductivity. Concerning this effect Walker (5) says: "The addition of a small quantity of a substance such as alcohol to water increases the viscosity of the water. Corresponding to this increase we find that the rate of diffusion is less when a substance is dissolved in water containing a little alcohol than the rate of diffusion when water alone is the solvent, no matter what the dissolved substance may be. Similarly the speed of ions in water containing alcohol is less than their speed in pure water."

Let us consider some of Osterhout's experiments more closely. He measured the conductivity of Laminaria in various solutions of anesthetics and compared it with the conductivity of the same material in sea-water. In one experiment he adds to 970 cc. sea-water 10 cc. of ether plus about 5 cc. of sea-water concentrated by evaporation until its conductivity was about double that of ordinary sea-water. In another experiment he adds to 970 cc. sea-water 30 cc. of absolute alcohol plus 15 cc. of concentrated sea-water (apparently made up as before), thus obtaining a 0.5 molecular solution of alcohol which he refers to as 0.05 molecular. This solution, according to Osterhout, has the same conductivity as sea-water. But this scarcely seems possible. Osterhout's solution has approximately the same concentration of salts as sea-water, but the viscosity of the solution is decidedly higher. Pissarjewsky and Karp (6) found that a 0.5 molecular concentration of alcohol lowers the conductivity of normal NaCl solution until it is about 8 per cent. below that of the conductivity of normal NaCl solution in pure water. On the other hand, the ether solution used by Osterhout would have, as he claims, approximately the same conductivity as sea-water, for Arrhenius (7) found that 1 per cent. ether lowers conductivity only about 2 per cent. for various types of electrolytes.

But there is also another important fact to be considered. Separating the cells of *Laminaria* is a network of cell walls. This is composed of cellulose and cellulose-like material which offers little resistance to dissolved substances and is obviously one of the main paths for an electric current. The adsorptive powers of cellulose are well known, although it apparently adsorbs electrolytes much more readily than non-polar compounds. In the finely divided condition in which the cellulose occurs in the cell walls it is not improbable that substances of low surface tension like ether, alcohol, and chloroform would be selectively adsorbed on it and more or less concentrated there. We should at least be led to expect this from Gibbs' adsorption equation. Moreover the surfaces of the cells would also tend to adsorb the anesthetic. If such adsorptive processes occur, then the conductivity of the cellulose framework as well as the conductivity of the cell surfaces would doubtless be decreased, and the decrease in conductivity would be greater than that which occurred in the mass of the solution. If this is true then it might in itself account for the variations in conductivity found by Osterhout.

It is interesting to note that the experiments of McClendon (8) on the diffusion of electrolytes from anesthetized pike eggs also fail to take cognizance of the direct effect of the anesthetic on the diffusion rate. It might be thought that the difference in diffusion rate would also explain McClendon's experiments. This is not the case. The presence of 2 per cent. alcohol would lower the diffusion rate less than 8 per cent. McClendon apparently found a lowering of as much as 50 per cent. in the total amount of chlorides diffused from the eggs. But his results are not as trustworthy as it might at first sight be thought. McClendon determined the diffused chlorides nephelometrically as silver chloride, precipitating them with silver nitrate. Now the precipitation of silver chloride varies under diverse conditions; there is a well known tendency for it to go into colloidal solution. The presence of alcohol either alone, or in conjunction with the albuminous substances also present, might act as a peptizing agent and hinder the precipitation of the silver chloride. Alcohol occasionally acts as a peptizing agent, and the precipitation of

silver chloride is known to be hindered by at least one non-electrolyte (see Bancroft (9), pp. 167, 168). Richard and Wells (10) in their first description of the nephelometer stated that "care must be taken to have both standard solution and unknown solution subjected to precisely the same conditions, for varying conditions of precipitation may lead to differences in the appearance of the precipitate far greater than the possible optical error of the apparatus. Herein lies the chief caution to be noted in its use." Later this point was emphasized again by Richards (11), who points out that "if even moderately accurate analytical results are to be had with the nephelometer, the one essential point to be heeded is this: *the unknown solutions to be estimated must be treated in exactly the same way as the known standard solutions, which serve as the basis for comparison*." This precaution was neglected by McClendon and his results are therefore unreliable.

McClendon's work and that of Osterhout constitute a large portion of the evidence presented by Winterstein and regarded by him as trustworthy evidence in favor of the permeability theory. It would seem therefore that the support of the theory does not always rest on very solid ground.

Most of the earlier work on permeability change during anesthesia concerned itself with the attempt to demonstrate a change in permeability toward dissolved substances. More recently both Winterstein (12) and Lillie (13) have independently shown a change in permeability to water. I have repeated both sets of experiments, although in the former case I performed only a few tests and my results can scarcely be considered as constituting either a proof or a disproof of Winterstein's views. Fig. 1 shows two examples of the entrance of water into normal and anesthetized muscle as shown by the increase in weight. The abscissas represent time, the ordinates the weight of the muscle. The muscle used was the gastrocnemius muscle of the frog, and a pair of muscles from a single animal was used in each experiment.

The curves show that the entrance of water is faster in the normal muscle than in the anesthetized muscle. Winterstein obtained a somewhat more striking difference; he used the sartorius muscle rather than the gastrocnemius.

As to the interpretation of the experiments, the difference in rate of osmotic flow may be due either to a change in the properties of the fluid or to a change in the plasma membranes. Bigelow (14) and Bartell (15) found that the speed of osmotic

FIG. 1 shows the increase in weight of muscles in distilled water and in distilled water to which 2 per cent. by volume of ether has been added. In each pair of curves the small circles represent the increase in weight of a frog gastrocnemius muscle in distilled water, the small triangles represent the increase in weight of the corresponding muscle from the other leg immersed in 2 per cent. ether. The abscissæ indicate time in hours, the ordinates total weight of muscle.

flow of water through various osmotic membranes followed the laws of Poseuille for flow through capillary tubes. It is obvious that in general any increase in the viscosity of a fluid would tend to slow its rate of osmotic flow. The magnitude of the effect might be sufficient to account for my results, although perhaps not for those of Winterstein. There are also other factors to be considered. The anesthetics may extract materials from the muscle which tend to exert osmotic pressure in the opposite direction.

I have thus far omitted reference to Winterstein's experiments on the osmotic flow through muscle membranes obtained from

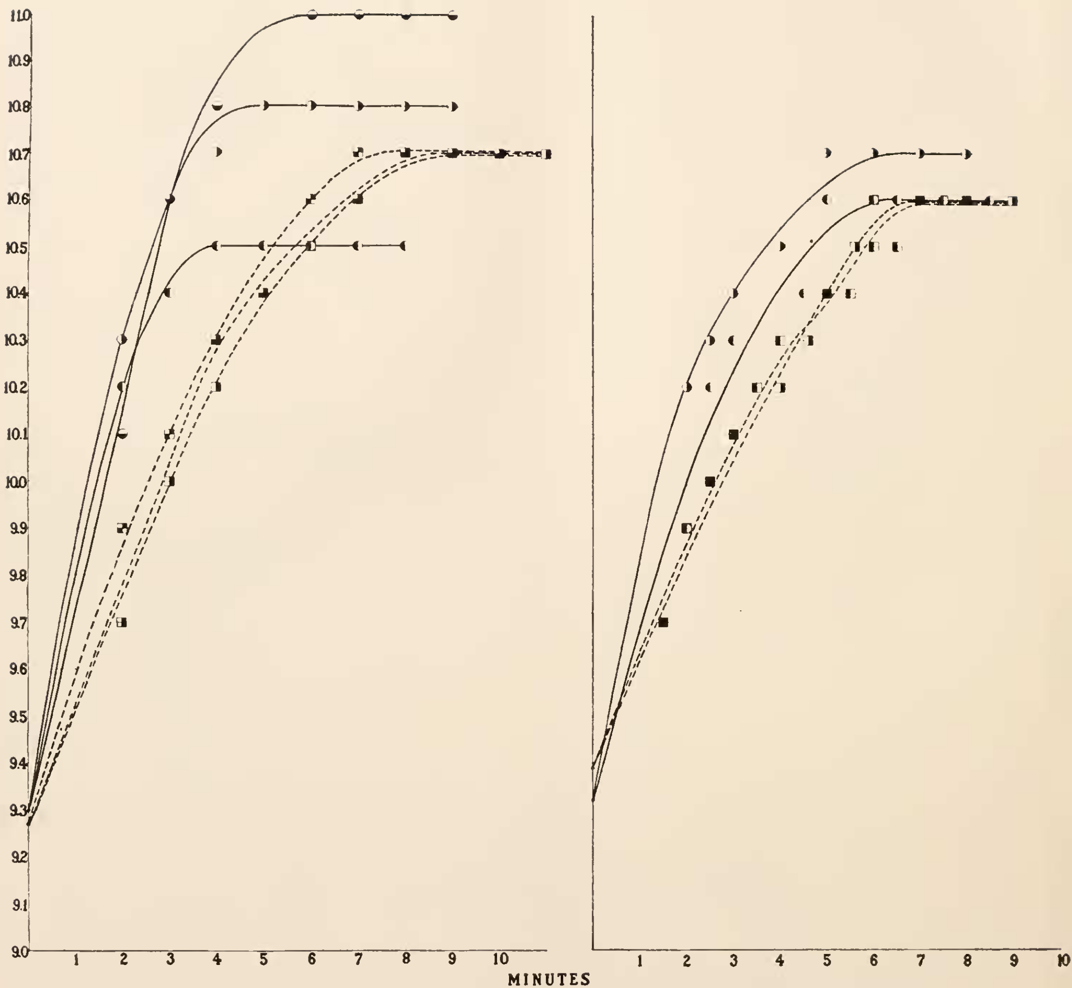


Fig. 2. In the above figures the curves represent increase of diameter of individual sea-urchin eggs in equal parts of sea-water and distilled water. (The original size is an average measurement.) The continuous lines represent etherized eggs, the broken lines control eggs, not exposed to ether. The concentration of ether used was 1 per cent. by volume, and the eggs were first treated with 1 per cent. ether in sea-water and were then transferred to 1 per cent. ether in equal parts of sea-water and distilled water. The ordinates represent the diameter of the eggs in arbitrary scale units, the abscissæ show the number of minutes elapsed after the entrance of the egg into the hypotonic solution. Similar results were also obtained with 2 per cent. ether solutions, but the measurements are harder to make as the eggs frequently rupture.

the body wall of female frogs. These experiments constitute the greater portion of Winterstein's evidence and the reader is referred to his paper for details. As I understand the experiments, Winterstein measured the osmotic flow into short glass cylinders sealed at *both* ends with muscle membranes. In such a system it would seem that the entrance of water would depend more on the distensibility of the muscle membranes than on their permeability. The high concentration of alcohol and other anesthetics used by Winterstein might tend to coagulate the membranes and make them less distensible. This would simulate a decreased permeability. However I have not worked with this sort of a set-up and I am not sure that I can judge it properly.

My results with sea-urchin eggs are perhaps more interesting. With this type of material Lillie found that the shrinkage of the cell in hypertonic solutions was delayed by the presence of ether in anesthetic concentration. This may very well be true, but it need not be due to a change in permeability to water. For it is easy to show that when eggs are placed in hypotonic solutions, they expand just as readily, or even more readily in the presence of ether than in its absence. This is illustrated in Fig. 2, which compares the expansion of etherized and unetherized eggs in hypotonic solutions. The shrinkage and expansion of a cell depends on many factors. Of these permeability is one, but not the only one. Other factors of importance are surface tension, the rigidity of the plasma membrane, and the colloidal condition of the interior. This last factor is of especial importance in Lillie's experiments. Lillie found that after fertilization the sea-urchin eggs showed a greater tendency to crenation and shrinkage when placed in hypertonic solutions, and that this increased tendency to crenation was prevented by anesthetics. As a matter of fact fertilized eggs become somewhat crenate even without being placed in hypertonic solutions (16). The crenation is concomitant with, and doubtless dependent on, the gelation of the protoplasm which follows fertilization. In view of the fact that anesthetics such as ether prevent this gelation (see below), it is easy to see that they would tend to hinder crenation.

Finally it should be pointed out that the experiments of Loeb (17) apparently indicate an *increase* in the permeability of *Fundulus* eggs to water in the presence of various anesthetics.

It is not my purpose to claim that in anesthesia there is no decrease in the permeability of the cell to salts or to water. Perhaps eventually this may be found to be a constant occurrence. But it seems at least premature to conclude a decreased permeability from the slight and uncertain evidence hitherto presented. Only by closing our eyes to the experiments that show the opposite of a decrease and only by neglecting the numerous sources of error which have scarcely been considered by previous experimenters, is it possible to consider the permeability evidence as convincing.

Even should we, some time in the future, find it true that anesthesia is always associated with decreased permeability of the plasma membrane of the cell, we would not be very much closer to an understanding of how and why the anesthetic stops cell activity. Permeability is a surface phenomenon, the activity of the cell goes on largely in the interior. Thus even if we accept the permeability doctrine, we must postulate a secondary hypothesis to explain how the permeability effect is transferred to the interior of the cell.

Many years before the permeability doctrine was thought of, various physiologists held the idea that anesthetics affected the colloidal condition of the protoplasm in one way or another. For a long time this idea remained a mere speculation, but in recent years it has been definitely shown that anesthetics do have a very real effect on the colloidal state, or at any rate upon the viscosity of the protoplasm. Both in plant and animal cells it has been shown that dilute solutions of ether cause a liquefaction of the protoplasm, and that more concentrated solutions cause a coagulation. There is a remarkable correspondence between widely different sorts of living substance. However in plants only those concentrations of ether which cause coagulation prevent the rotary movement of the protoplasm. This was therefore regarded by Heilbronn (18) as the anesthetic concentration. In animal cells on the other hand it was found that concentrations which caused liquefaction prevented cell-

division and therefore acted as anesthetic (16). As a matter of fact there is no difficulty in reconciling the two sets of observations. For it seems certain that what is anesthetic for mitosis is not necessarily anesthetic for protoplasmic streaming. An increased fluidity of the protoplasm would doubtless increase the speed of streaming, and yet such a cell with rapidly flowing protoplasm would scarcely be able to undergo a mitotic division and would therefore be anesthetized so far as cell-division was concerned. This is essentially the view taken by Weber (19) who points out that there is full accord in the investigation of both plant and animal cells.

Doubtless protoplasmic activity involves frequent changes in colloidal condition. Presumably any agent which prevents such changes in colloidal condition without causing death may act as an anesthetic. If this view is correct then either an increased liquefaction or a coagulation of the protoplasm may result in anesthesia. This idea was presented a number of years ago, and it was pointed out that there may very well be two kinds of anesthesia (20).

In earlier work it was claimed that those solutions of ether which act as an anesthetic for the process of cell-division in the sea-urchin egg cause a liquefaction of the protoplasm (16, 20). More concentrated solutions were found to cause a coagulation which was irreversible. All these facts have recently been called into question by Chambers (21). He claims that anesthesia in the sea-urchin egg is accompanied by an *increase* rather than a decrease in viscosity and that this condition of increased viscosity is reversible. Chambers' opinion is based on observations of Brownian movement as well as on a study with the microdissection apparatus.

It is evident that the findings of Chambers are in direct opposition to the earlier work mentioned above. An attempt was therefore made to repeat the older observations with the centrifuge method to determine if perhaps an error may not have been involved. In this repetition an effort was made to obtain more nearly quantitative results.

Experiments were performed both with fertilized and unfertilized eggs. We will consider the unfertilized eggs first.

When (unfertilized) sea-urchin eggs are placed in 2½ per cent. or 3 per cent ether, there is, within a few minutes, a very pronounced decrease in viscosity as shown by tests with the centrifuge. In the following table the viscosity of etherized and normal control eggs is compared. The viscosity numbers represent the number of seconds of centrifugal treatment necessary to produce a given degree of granular movement. Usually the eggs were centrifuged until the clear hyaline zone extended along one third or one half of the axis of the egg. Naturally a number of tests had to be performed to obtain any given value. The centrifugal force employed was approximately 4,968 times gravity and the centrifuge used was an ordinary hand centrifuge with hæmatocrit attachment. Details of technique are given in earlier papers (16, 22). Because of the fact that a series of tests had to be performed for each value of the viscosity and because moreover the viscosity of the etherized protoplasm is not constant, the tests had to be made hurriedly. They are not extremely accurate, and it is entirely possible that the inaccuracy amounts to 10 or 20 per cent. The decided difference between unetherized and etherized eggs makes this inaccuracy of small consequence. In the table the first column gives the percentage of ether used (volume per cent. in sea-water). The second column represents the time of exposure to ether, that is to say the number of minutes elapsed after immersion in ether solution at the time the critical test was made. The third column gives the relative viscosity of the etherized eggs as compared to that of the normal control eggs shown in the fourth column. The temperature of the experiments varied from 22 to 25 degrees. It is given in the fifth column.

| Per Cent. Ether. | Exposure, Minutes. | Viscosity Etherized Eggs. | Viscosity Control Eggs. | Temperature. |
|---|---|---|---|---|
| 2.5 | 11 | 10 | 25 | 23° |
| 2.5 | 4 | 15 | 25 | |
| 2.5 | 8 | 15 | 25 | 22 |
| 2.5 | 10 | 15 | 28 | 24 |
| 3 | 15 | 15 | 35 | 22 |
| 3 | 8.5 | 20 | 40 | 23 |
| 3 | 3 | 15 | 30 | 25.3 |

Averaging the above values it is seen that in 2½ per cent.

ether the viscosity of the protoplasm is 53 per cent. of that of the normal control eggs in sea-water. The viscosity of the eggs in 3 per cent. ether is only 48 per cent. of that of the untreated eggs.

It must not be supposed that the viscosity of the protoplasm of etherized eggs remains constant for long periods. In the 3 per cent. solution the viscosity became lower and lower until finally a minimum was reached. Then a sudden coagulation occurred. This is shown by a series of tests on eggs in 3 per cent. ether (temp. 22.0°). When these eggs were tested after an exposure of 3 minutes it was found that centrifugal treatment for 15 seconds produced only a slight hyaline zone. The same results were obtained after 6 and 10 minutes, but after an exposure of 15 minutes, similar centrifugal treatment resulted in the formation of a hyaline zone which extended along about one third of the axis of the egg. After an exposure of 20 minutes the same treatment resulted in a hyaline zone which filled nearly half of the egg. On the other hand after 26 minutes when eggs were centrifuged for 15 seconds, although some eggs showed a hyaline zone extending through half of the egg, others showed no movement of granules at all. The protoplasm in these eggs is completely coagulated. Thus in 3 per cent. ether at 22° the liquefaction of the protoplasm is soon followed by a solidification.

The question may now be asked as to which of these two conditions represents a condition of anesthesia. In my earlier paper I had claimed that only the fluid state was anesthetic and that gelation or coagulation following ether treatment resulted only in death. But Chambers states that not the fluid but the gel state is the anesthetic condition when eggs are subjected to ether. On reading Chambers' statement I was at first led to suppose that I had been mistaken in making my claim too forcibly. I thought perhaps that although it was certain that moderately long exposure to ether after coagulation had taken place was lethal, nevertheless it might be true that Chambers had removed the eggs immediately after coagulation had begun and that in this instance the coagulative action of the ether was reversible.

The question was soon put to the test. In the experiment

cited above it was pointed out that coagulation occurred after 26 minutes exposure to ether. In this same experiment some of the eggs were removed from the ether solution after an exposure of 24 minutes and placed in normal sea-water. Others were removed from the ether after an exposure of 28 minutes. Some of the eggs exposed to ether 24 minutes were inseminated, following an interval of 18 minutes after removal from the ether, and some of the eggs exposed 28 minutes were inseminated following an interval of 14 minutes after removal from ether. None of the inseminated eggs showed any signs of development. As a matter of fact both the inseminated eggs and those not exposed to sperm went through the same series of degenerative changes. All of them disintegrated by breaking up into small globules.

Thus it is obvious that following the coagulative action of ether there is no recovery. The same sort of experiment was repeated a number of times always with the same result. If eggs are to recover from ether treatment they must be removed from the ether solution some few minutes before coagulation has begun.

The discussion so far has been concerned only with conditions in unfertilized eggs. In fertilized eggs the effects of ether are even more pronounced. Let us consider a sample experiment. In the following account many details of observation are omitted.

*July 22* (Temp. about 24°). Eggs were fertilized at 3.35 P.M. Fifteen minutes later, at 3.50 P.M., they were centrifuged at the usual rate for 50 seconds. No zones appeared (control unfertilized eggs showed a hyaline zone about ¼ of the distance along the egg axis after 30 seconds treatment). At 3.52 P.M. some of the fertilized eggs were placed in 2½ per cent. ether in sea-water in a glass-stoppered weighing bottle. At 3.55 P.M. a centrifugal test for 60 seconds showed only a thin streak for a hyaline zone in the control untreated fertilized eggs. At 4.27 P.M. the etherized eggs when centrifuged for 20 seconds showed a hyaline zone extending more than a third of the distance along the egg axis. At 4.31 P.M. a test of the etherized eggs for 15 seconds showed a similar zone extending about one third of the axis, and at 4.33 P.M. a 10 second test showed a hyaline zone extending through approximately one fourth of the egg.

From this experiment we can conclude that when fertilized eggs are placed in 2½ per cent. ether at a time when the viscosity of their protoplasm is at its height, the ether reduces the viscosity to less than one sixth of its original value. Another experiment of the same sort may also be cited.

*July 23* (Temp. 23°). Eggs were fertilized at 10.45 A.M. At 11.00 A.M. some of the fertilized eggs were put into 2½ per cent. ether in a glass-stoppered weighing bottle, others of the same lot were centrifuged for 60 seconds. The centrifugal treatment for 60 seconds resulted in a faint indication of a hyaline zone. At 11.05 A.M. a centrifugal test of the normal fertilized eggs for 80 seconds showed a hyaline zone extending about one fourth of the egg axis. Test samples of etherized eggs were centrifuged for 5 seconds at 11.12 A.M., for 10 seconds at 11.18 A.M., and for 15 seconds at 11.23 A.M. The 5 second test showed only faint indications of a hyaline zone, the 10 second test showed the zone not very plainly, the 15 second test showed it extending along one third of the axis of the egg. Later a 10 second test, at 11.35 A.M., showed the hyaline zone extending for ¼ to ⅓ of the egg axis. This test is not as trustworthy as the earlier tests, for the protoplasm appeared to be in chunks, and it is possible that the granules were fusing together. At 11.40 P.M., a 15 second test showed the hyaline zone ⅓, ¼, or absent. Coagulation is doubtless beginning.

This experiment showed that 2½ per cent. ether may lower the protoplasmic viscosity to one sixth or even one eighth of its original value in fertilized eggs.

As with the unfertilized eggs the protoplasm of the etherized fertilized eggs becomes more and more fluid with increase in ether concentration or length of exposure until suddenly coagulation occurs. Here too the onset of coagulation results in death. If the eggs are removed from the ether in the early stages of liquefaction they resume their development, but if they are kept in ether until coagulation has occurred or is about to occur, the eggs are permanently injured and never resume development.

The result of these experiments is a direct confirmation of my older results. These findings do not agree with the statement of Chambers previously referred to. Let us consider this statement closely. He says:

"Both A. Heilbronn and Weber agree with the coagulation theory of narcosis. On the other hand, L. Heilbrunn (1920) claims that the reversible effect of 2.5 per cent. ether on the sea-urchin egg occurs only when the viscosity is diminished. With higher concentrations of ether (3 per cent. +) the increased viscosity, according to him, is irreversible. He therefore concludes that narcosis implies a diminution in viscosity of the protoplasm. My results with the micro-dissection method and by observing Brownian movement do not agree with this. In 2 per cent. ether, Brownian movement was slowed down but

did not cease, and cleavage delayed but not stopped. In 2½ per cent. ether, cleavage was stopped and, both by means of the needle and by the cessation of Brownian movement, this was shown to be accompanied by a decided increase in viscosity (cf. p. 300)."

The first sentence of the above quotation is not quite true. As already pointed out Weber's ideas on ether narcosis agree exactly with mine. But this is a minor point. The essential fact is that Chambers has apparently obtained actual experimental results in direct contradiction with mine. How can this be interpreted?

In a previous paper I have already taken occasion to criticize the microdissection method for its over-great subjectivity (22), and this criticism has been supported by Heilbronn (23). But Chambers claims that his results were obtained both by microdissection and by the observation of Brownian movement. For some time I was puzzled as to how to explain the difference in his findings. Finally the idea suggested itself that Chambers in his studies of Brownian movement may have subjected the eggs to heat. It has already been shown that heat and ether act together, so that in low concentrations of ether, heat coagulation is hastened (24). Such coagulation is moreover reversible. On questioning Chambers it was found that in his experiments an arc lamp was used for illumination and no special precaution was taken to eliminate the heat factor. Here then is an explanation of the divergent results obtained by the Brownian movement method. As for Chambers' results with the microdissection method, these are probably not very trustworthy, for he was doubtless influenced by the results of his Brownian movement investigations. Another worker, Hyman, also using the dissection method, has roughly confirmed my views concerning the effects of ether on the protoplasm of sea-urchin eggs (25). Miss Hyman used ordinary steel needles instead of a microdissection apparatus.

## Summary.

1. Water enters etherized muscle less rapidly than normal muscle, but this does not necessarily imply a change in the properties of the plasma membrane following etherization.

2. Ether does not lower the permeability of sea-urchin eggs to water.

3. Dilute ether solutions cause a very sharp decrease in the viscosity of sea-urchin egg protoplasm, both in fertilized and unfertilized eggs. Rough quantitative measurements of this decrease are given.

4. Slightly more concentrated solutions of ether produce a coagulation which is irreversible.

5. The divergent results of Chambers find a simple explanation.

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OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

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PUBLISHED MONTHLY BY THE

MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY

LANCASTER PRESS, INC.

LANCASTER, PA.

AGENT FOR GREAT BRITAIN

WHELDON & WESLEY, LIMITED

*2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2*

Single Numbers, $1.00. Per Volume (6 numbers), $4.50

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

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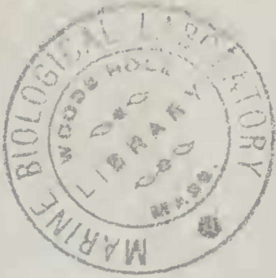
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OF THE

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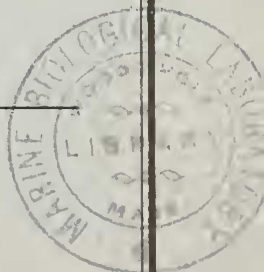



Published Monthly by the

MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY

LANCASTER PRESS, Inc.

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# BIOLOGICAL BULLETIN

OF THE

**Marine Biological Laboratory**

WOODS HOLE, MASS.

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PUBLISHED MONTHLY BY THE

MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY

LANCASTER PRESS, INC.
LANCASTER, PA.

AGENT FOR GREAT BRITAIN

WHELDON & WESLEY, LIMITED

*2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2*

**Single Numbers, $1.00. Per Volume (6 numbers), $4.50**

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894

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Published Monthly by the

MARINE BIOLOGICAL LABORATORY

printed and issued by

LANCASTER PRESS, Inc.

LANCASTER, PA.

Agent for Great Britain

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